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(54) Title: METHODS OF IDENTIFYING MOLECULES THAT HOME TO ANGIOGENIC VASCULATURE IN TUMORS		
(57) Abstract A method of identifying a tumor homing molecule that homes to angiogenic vasculature by contacting a substantially purified NGR receptor with one or more molecules and determining specific binding of a molecule to the NGR receptor, where the presence of specific binding identifies the molecule as a tumor homing molecule that homes to angiogenic vasculature. The invention also provides a method of directing a moiety to angiogenic vasculature in a subject by administering to the subject a conjugate including a moiety linked to a tumor homing molecule that exhibits specific binding to an NGR receptor, whereby the moiety is directed to angiogenic vasculature. In addition, the invention provides a method of imaging the angiogenic vasculature of a tumor in a subject by administering to the subject a conjugate having a detectable moiety linked to a tumor homing molecule that exhibits specific binding to an NGR receptor and detecting the conjugate.		

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METHODS OF IDENTIFYING MOLECULES THAT HOME TO ANGIOGENIC VASCULATURE IN TUMORS

This application is a continuation-in-part of U.S. Application 08/926,914, filed September 10, 1997, which
5 claims the benefit of priority of U.S. Provisional Application No. 60/060,947, which was converted from United States Serial No. 08/710,067, filed September 10, 1996, the entire contents of which is incorporated herein by reference.

10 This invention was made with government support under CA 42507, CA 62042, CA74238-01 and Cancer Center Support Grant CA 30199 awarded by the National Institutes of Health. The government has certain rights in this invention.

BACKGROUND OF THE INVENTION15 FIELD OF THE INVENTION

The present invention relates generally to the fields of cancer biology and drug delivery and, more specifically, to peptides that selectively home to a tumor, particularly a malignant tumor, to compositions comprising an agent such as
20 a therapeutic agent conjugated to such tumor homing molecules, and to methods of using such molecules to target an agent to a tumor.

BACKGROUND INFORMATION

Continuous developments over the past quarter century
25 have resulted in substantial improvements in the ability of a physician to diagnose a cancer in a patient. For example,

antibody based assays such as that for prostate specific antigen now allow early diagnosis of cancers such as prostate cancer. More recently, methods of genetic screening are becoming available to identify persons that
5 may be particularly susceptible to developing a cancer. Genetic screening methods are based on the identification of one or more mutations in a gene that correlates with the development of a cancer. For example, the identification of genes such as BRCA1 and BRCA2 allowed the further
10 identification of mutations in these genes that, in some cases, can correlate with susceptibility to developing breast cancer.

Unfortunately, methods for treating cancer have not kept pace with those for diagnosing the disease. Thus,
15 while the death rate from various cancers has decreased due to the ability of a physician to detect the disease at an earlier stage, the ability to treat patients presenting with more advanced disease has advanced only minimally.

A major hurdle to advances in treating cancer is the
20 relative lack of agents that can selectively target the cancer, while sparing normal tissue. For example, radiation therapy and surgery, which generally are localized treatments, can cause substantial damage to normal tissue in the treatment field, resulting in scarring and, in severe
25 cases, loss of function of the normal tissue. Chemotherapy, in comparison, which generally is administered systemically, can cause substantial damage to organs such as bone marrow, mucosae, skin and the small intestine, which undergo rapid cell turnover and continuous cell division. As a result,
30 undesirable side effects such as nausea, loss of hair and

drop in blood cell count occur as a result of systemically treating a cancer patient with chemotherapeutic agents. Such undesirable side effects often limit the amount of a treatment that can be administered. Thus, cancer remains a leading cause of patient morbidity and death.

Efforts have been made to increase the target specificity of various drugs. For example, where a unique cell surface marker is expressed by a population of cells making up a tumor, an antibody can be raised against the unique marker and a drug can be linked to the antibody. Upon administration of the drug/antibody complex to the patient, the binding of the antibody to the marker results in the delivery of a relatively high concentration of the drug to the tumor. Similar methods can be used where a particular cancer cell or the supporting cell or matrix expresses a unique cell surface receptor or a ligand for a particular receptor. In these cases, the drug can be linked to the specific ligand or to the receptor, respectively, thus providing a means to deliver a relatively high concentration of the drug to the tumor.

Tumors are characterized, in part, by a relatively high level of active angiogenesis, resulting in the continual formation of new blood vessels to support the growing tumor. Such angiogenic blood vessels are distinguishable from mature vasculature. One of the distinguishing features of angiogenic vasculature is that unique endothelial cell surface markers are expressed. Thus, the blood vessels in a tumor provide a potential target for directing a chemotherapeutic agent to the tumor, thereby reducing the likelihood that the agent will kill sensitive normal

tissues. Furthermore, if agents that target the angiogenic blood vessels in a tumor can be identified, there is a likelihood that the agents can be useful against a variety of different types of tumors, since it is the target
5 molecules in the angiogenic vessels that are recognized by such agents and not receptors specific for the tumor cells. However, the use of molecules that can bind specifically to tumor vasculature and target a chemotherapeutic agent to the tumor has not been demonstrated.

10 While linking a drug to a molecule that homes to a tumor can provide significant advantages for treatment over the use of a drug, alone, use of this method is severely limited by the scarcity of useful cell surface markers expressed in a tumor. Thus, a need exists to identify
15 molecules that can selectively home to a tumor, particularly to the vasculature supporting the tumor. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

20 The present invention provides a method of identifying a tumor homing molecule that homes to angiogenic vasculature of a tumor. The method includes the steps of contacting a substantially purified NGR receptor with one or more molecules and determining specific binding of a molecule to
25 the NGR receptor, where the presence of specific binding identifies the molecule as a tumor homing molecule that homes to angiogenic vasculature of a tumor. In a method of the invention, the substantially purified NGR receptor can be, for example, CD13/aminopeptidase N. If desired, the

substantially purified NGR receptor can be immobilized on a support such as a plate or a bead.

The invention also provides a method of identifying a homing molecule that homes to angiogenic vasculature using substantially purified NGR receptor. The method includes the steps of contacting a substantially purified NGR receptor with one or more molecules and determining specific binding of a molecule to the NGR receptor, where presence of specific binding identifies the molecule as a homing molecule that homes to angiogenic vasculature. The invention provides homing molecules that home to non-tumor angiogenic vasculature.

The present invention also provides a method of directing a moiety to angiogenic vasculature of a tumor in a subject by administering to the subject a conjugate including a moiety linked to a tumor homing molecule that exhibits specific binding to an NGR receptor, whereby the moiety is directed to angiogenic vasculature of a tumor. In a method of the invention, the tumor homing molecule can be, for example, a peptide containing the sequence NGR, and, if desired, can be part of a conjugate in which the moiety is a cytotoxic agent, drug or cancer therapeutic agent, for example, doxorubicin. A tumor homing peptide containing the sequence NGR can have, for example, the sequence CNGRCVSGCAGRC (SEQ ID NO:3), NGRAHA (SEQ ID NO:6), CVLNGRMEC (SEQ ID NO:7) or CNGRC (SEQ ID NO:8). In a method of the invention for directing a moiety to angiogenic vasculature of a tumor in a subject, the tumor homing molecule also can be, for example, an aminopeptidase inhibitor such as bestatin, o-phenanthroline, actinonin, amastatin, 2,2'-

dipyridyl or fumagillin and can be linked, if desired, to a drug moiety.

Further provided herein is a method of imaging the angiogenic vasculature of a tumor in a subject by
5 administering to the subject a conjugate having a detectable moiety linked to a tumor homing molecule that exhibits specific binding to an NGR receptor, whereby the conjugate selectively binds the angiogenic vasculature, and detecting the conjugate. A detectable moiety for imaging angiogenic
10 vasculature can be, for example, a radionuclide. A useful tumor homing molecule for imaging angiogenic vasculature can be, for example, a peptide containing the sequence NGR, such as a peptide containing the sequence CNGRCVSGCAGRC (SEQ ID NO:3), NGRAHA (SEQ ID NO:6), CVLNGRMEC (SEQ ID NO:7) or
15 CNGRC (SEQ ID NO:8). A tumor homing molecule for imaging angiogenic vasculature also can be, for example, an aminopeptidase inhibitor such as bestatin, o-phenanthroline, actinonin, amastatin, 2,2'-dipyridyl, fumagillin or another molecule that inhibits an aminopeptidase.

20 The invention also provides inhibitors of angiogenesis that are NGR receptor binding molecules. Such inhibitors can be, for example, an NGR receptor antibody or an aminopeptidase inhibitor such as bestatin, o-phenanthroline, actinonin, amastatin, 2,2'-dipyridyl or fumagillin, or a
25 conjugate of such angiogenesis inhibitors to a drug or toxin.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows inhibition of *in vivo* phage homing by synthetic peptides. Recovery of phage displaying tumor homing peptides from breast carcinoma xenografts was measured after injection of phage or coinjection of the phage with various peptides. (A) Left panel: Recovery of phage expressing the NGR tumor homing peptide, CNGRCSVSGCAGRC (SEQ ID NO:3; "NGR phage") from tumor (filled bars) and brain (striped bars), and inhibition of the tumor homing by the soluble peptide CNGRC (SEQ ID NO:8). Middle panel: Recovery of CGSLVRC-phage and inhibition of the tumor homing by the soluble peptide CGSLVRC. Right panel: Recovery of RGD-4C phage (positive control; the peptide insert in the RGD-4C phage is CDCRGDCFC; SEQ ID NO:1) and unselected phage library mix (negative control). (B) Left panel: Increasing amounts of the RGD-4C soluble peptide were injected with the CNGRCSVSGCAGRC-phage. Right panel: Increasing amounts of the CNGRC soluble peptide were injected with the RGD-4C phage.

Figure 2 shows the specificity of tumor homing by the NGR phage relative to the positive control (RGD-4C) and negative control (fd-tet) phage.

Figures 3A to 3V show the immunohistochemical staining of the NGR phage in tumors and normal tissues following intravenous injection into nude mice bearing a human breast carcinoma or a human Kaposi's sarcoma. Samples were taken 4 min (Figures 3E, 3G, 3H and 3J) or 24 hr (Figures 3A to 3D, 3F, 3I, and 3K to 3V) after administration of the phage. Figures 3A, 3C, 3G and 3J are from mice receiving insertless phage (control phage) and Figures 3B, 3D, 3E, 3F, 3H, 3I and

3K to 3V are from mice receiving NGR phage. Figures 3A, 3B, 3E, 3F and 3G are breast tumor samples; Figures 3C, 3D, 3H, 3I and 3J are Kaposi's sarcoma samples; Figure 3K is brain; Figure 3L is lymph node; Figure 3M is kidney; Figure 3N is pancreas; Figure 3O is uterus; Figure 3P is mammary fat pad; Figure 3Q is lung; Figure 3R is intestine; Figure 3S is skin; Figure 3T is skeletal muscle; Figure 3U is heart and Figure 3V is urinary tract epithelium. Magnification: Figures 3A to 3D, 40x; Figures 3E to 3V, 200x.

10 Figure 4 shows isolation of CD13/aminopeptidase N (APN) and CNGRC-phage binding to CD13/APN. (A) Aminopeptidase enzymatic activity in immuno-isolated CD13 detected by using the CD13 substrates Ala-pNA or Leu-pNA. (B) Phage binding to immuno-isolated CD13 from a Kaposi's sarcoma tumor cell
15 octylglucoside extract. Phage carrying the indicated peptides were tested for binding to CD13 that was immobilized using an anti-CD13 antibody WM15 coated on microtiter wells. (C) Inhibition of NGR-phage binding to CD13 by an NGR-containing cyclic peptide (CNGRC; SEQ ID
20 NO:8) but not by an RGD-containing cyclic peptide or by an unrelated peptide.

Figure 5 shows binding of phage expressing an NGR containing peptide to cells transfected with CD13. CNGRC-phage binding to control Molt-4 cells and to Molt-4 cells
25 transfected with CD13 and inhibition of the binding by the CNGRC cyclic peptide.

Figure 6 shows affinity purification of CD13 from HL-60 cell extracts using an NGR peptide column and elution with CNGRC peptide. CD13 was detected in the fractions by using

the WM15 monoclonal antibody, and normal mouse IgG was used as a negative control. Samples of each fraction were analyzed for aminopeptidase enzymatic activity by using Ala-PNA, and for matrix metalloprotease (MMP) activity by
5 using an MMP substrate.

Figure 7 shows upregulation of CD13 in cultured cells by angiogenic factors. Upregulation of CD13 by angiogenic factors in human umbilical cord endothelial cells (HUVEC).

Figure 8 shows CD13-dependent cytotoxic activity of
10 doxorubicin/CNGRC (CNGRC-dox) *in vitro*. (A) CD13-dependent cytotoxic activity of CNGRC-doxorubicin *in vitro* in activated HUVECs. (B) CD13-dependent activity of CNGRC-doxorubicin *in vitro* in CD13 transfected MDA-MB-435 breast carcinoma cells.

15 Figure 9 shows recovery of CNGRC phage, RGD-4C phage, and control phage (insertless fd) from breast carcinoma xenografts, normal retina and angiogenic retina. (A) The experimental design is shown. (B) The ratio of recovered tetracycline-resistant to ampicillin-resistant phage is
20 shown.

Figure 10 shows the suppression of bFGF-induced angiogenesis in chicken chorioallantoic membrane and inhibition of tumor growth by CD13 antagonists. (A) CD13 antagonists, anti-CD13 antibody, bestatin and actinonin,
25 suppress bFGF-induced angiogenesis. (B) Inhibition of the growth of 435 breast carcinoma tumors upon injection of a CD13 antagonist antibody.

Figure 11 shows treatment of mice bearing MDA-MB-435-derived breast carcinomas and Hodgkin's lymphoma with doxorubicin-CNGRC peptide conjugate. Mice with size-matched tumors ($\sim 1000 \text{ mm}^3$) were randomized into four treatment groups (six animals per group): vehicle only, free doxorubicin (dox), doxorubicin-control peptide (dox-ctrl pep), and doxorubicin-CNGRC (dox-CNGRC). (A) Mice were treated at 5 $\mu\text{g}/\text{mouse}/\text{week}$ of doxorubicin-equivalent. Difference in tumor volumes between day 1 and day 28 are shown. (B) A Kaplan-Meier survival curve of the mice in Panel A is shown. (C) Mice bearing large ($\sim 5000 \text{ mm}^3$) MDA-MB-435 breast carcinomas (four animals per group) were randomized to receive a single-dose of free doxorubicin or doxorubicin-CNGRC conjugate at 200 $\mu\text{g}/\text{mouse}$ of doxorubicin equivalent. A Kaplan-Meier survival curve is shown. (D) Mice bearing large ($\sim 5000 \text{ mm}^3$) Hodgkin's lymphoma (eight animals per group) were randomized to receive two doses of free doxorubicin plus unconjugated CNGRC peptide or doxorubicin-CNGRC conjugate at 40 $\mu\text{g}/\text{mouse}$ of doxorubicin equivalent. A Kaplan-Meier survival curve is shown.

Figure 12 shows the nucleotide and amino acid sequences of CD13/aminopeptidase N (SEQ ID NOS:200 and 201, respectively).

DETAILED DESCRIPTION OF THE INVENTION

25 The present invention relates to the identification of a target molecule responsible for the homing of molecules to angiogenic vasculature. The identified target molecule can act as a receptor, for example, for tumor homing molecules that home to the angiogenic vasculature of a tumor. As

disclosed herein, various tumor homing molecules were isolated using *in vivo* panning; a core binding motif present in several of the tumor homing peptides was identified as the sequence NGR (see Example IV). In particular, phage
5 expressing the peptides CNGRCSVSGCAGRC (SEQ ID NO:3), NGRAHA (SEQ ID NO:6) and CVLNGRMEC (SEQ ID NO:7) homed to human breast carcinomas, human Kaposi's sarcomas and mouse melanomas in mice bearing these tumors. Furthermore, such homing was competitively inhibited *in vivo* by the NGR
10 containing peptide CNGRC (SEQ ID NO:8) but not by an unrelated peptide. Thus, the results disclosed herein indicate that a tumor homing peptide containing NGR can home to and specifically bind tumors of different types and species origin.

15 As further disclosed herein, a receptor that specifically binds tumor homing molecules containing the NGR motif has been identified (see Examples IX and X). Characterization of the NGR receptor revealed that this molecule immunoreacts with CD13 antibodies and that the
20 isolated receptor specifically binds the NGR motif. Furthermore, the NGR receptor was expressed in angiogenic vasculature, including tumor vasculature, and is functionally important in angiogenesis. The identification of a target molecule such as the NGR receptor that is
25 expressed in angiogenic vasculature can be advantageously used *in vitro* to identify new homing molecules, including high affinity ligands of the NGR receptor, as well as to target a tumor homing molecule and a linked moiety, such as a drug, to the angiogenic vasculature of a tumor *in vivo*.

Thus, the present invention provides a method of identifying a tumor homing molecule that homes to angiogenic vasculature of a tumor by using a substantially purified NGR receptor to identify the molecule. The method includes the steps of contacting a substantially purified NGR receptor with one or more molecules and determining specific binding of a molecule to the NGR receptor, where the presence of specific binding identifies the molecule as a tumor homing molecule that homes to angiogenic vasculature of a tumor. A method of the invention directed to identifying a tumor homing molecule that homes to angiogenic vasculature of a tumor can additionally include the steps of administering an NGR binding molecule *in vivo* and determining binding of the NGR binding molecule to angiogenic vasculature. If desired, the substantially purified NGR receptor can be immobilized on a support such as a plate or a bead.

In a method of the invention, the substantially purified NGR receptor can be, for example, CD13/aminopeptidase N (Figure 12; see, also, Look et al., J. Clin. Invest. 83:1299-1307 (1989), which is incorporated herein by reference). This highly conserved transmembrane glycoprotein of about 150 kDa is incorporated into the cell membrane through an N-terminal hydrophobic segment (Look et al., *supra*, 1989; Xu et al., Exp. Hematol. 25:521-529 (1997), which is incorporated herein by reference). The large extracellular carboxy-terminal domain contains a pentapeptide that is characteristic of many zinc-dependent metalloproteases (Look et al., *supra*, 1989). Homologs of CD13 from several different species are well conserved (Look et al., *supra*, 1989; Xu et al., *supra*, 1997; Turner et al.,

in Mammalian Ectoenzymes, Kenny and Turner, eds., Elsevier Scientific Publishing Co., Amsterdam, p. 211 (1987)).

CD13 is expressed in normal and malignant cells of the myeloid lineage (Amoscato et al., J. Immunol. 142:1245-1252
5 (1989); Favaloro et al., Br. J. Haematol. 69:163-171 (1988); Makrynika et al., Exp. Hematol. 23:1173-1179 (1995)) as well as in many epithelial, endothelial, and tumor cell types (Amoscato et al., Biochem. Biophys. Acta 1041:317-319 (1990); Rawlings and Barret, Biochem. J. 290:205-218 (1993);
10 Mechttersheimer and Moller, Am. J. Pathol. 137:1215-1222 (1990); Menrad et al., Cancer Res. 53:1450-1455 (1993); Riemann et al., J. Immunol. 158:3425-3432 (1997)). CD13 can function differently depending on its location. In synaptic membranes, CD13 metabolizes enkephalins and endorphins
15 (Matsas et al., FEBS Lett. 175:124-128 (1984)); in the intestinal brush border, it degrades regulatory peptides and scavenges amino acids (Turner et al., *supra*, 1997; Rawlings and Barret, *supra*, 1993); in lymphocytes, the cell surface activity of CD13 is associated with mitotic activation,
20 antigen processing (Mouritsen et al., J. Immunol. 149:1987-1993 (1992); Falk et al., Immunogenetics 39:230-242 (1994)), cell adhesion, and migration (Menrad et al., *supra*, 1993; Saiki et al., Int. J. Cancer 54:137-143 (1993); Koch et al., Am. J. Pathol. 138:165-173 (1991)). In addition, CD13 has
25 also been implicated in tumor invasion (Saiki et al., *supra*, 1993; Fujii et al., Clin. Exp. Metastasis 13:337-344 (1995)), signal transduction (O'Connell et al., Transplant. Proc. 21:3826-3827 (1989)), cell cycle control and differentiation (Makrynika et al., *supra*, 1995; Riemann
30 et al., *supra*, 1997), and as a receptor for viruses (Delmas

et al., Nature 357:417-420 (1992); Yeager et al., Nature 357:420-422 (1992)).

The expression levels and enzymatic activity of CD13 can be physiologically regulated, with the activity and
5 substrate specificity of CD13 correlating with conformational changes and induced by various stimuli such as proliferative signals to cells. Studies using monoclonal antibodies also have indicated that CD13 undergoes
regulatory intramolecular alterations that can result in the
10 exposure of cryptic sites and can regulate enzyme activity. The presence of certain epitopes has also been related to prognosis of acute myeloid leukemia (Xu et al., *supra*, 1997; Favalaro et al., *supra*, 1988; Makrynika et al., *supra*, 1995).

15 Cell-surface CD13/aminopeptidase N enzymatic activity can be potently blocked by bestatin, o-phenanthroline and actinonin (Taylor, FASEB J. 7:290-298 (1993); Rawlings and Barret, *supra*, 1993; Saiki et al., *supra*, 1993). Moreover, bestatin has been shown to possess immunomodulatory effects,
20 and administration of high doses of bestatin results in marked suppression of experimental and spontaneous metastasis and inhibition of tumor cell invasion (Bruley-Rosset et al. Immunol. 38:75-83 (1979); van Hal et al., J. Immunol. 153:2718-2728 (1994); Saiki et al., *supra*, 1993;
25 Fujii et al., *supra*, 1995)). Although CD13 is expressed outside the vascular system, the results disclosed herein indicate that an NGR receptor having immunoreactivity with an anti-CD13 antibody is only exposed to the circulation in tumor vessels (see Example XII).

As used herein, the term "NGR receptor" means a target molecule that is expressed in angiogenic vasculature and that specifically binds an NGR motif. As described below and in Examples IX and X, an NGR receptor has been

5 substantially purified and demonstrated to specifically bind several NGR containing peptides but not unrelated control peptides. The NGR receptor disclosed herein exhibits characteristics of a highly conserved transmembrane aminopeptidase designated CD13/aminopeptidase N (CD13/APN).

10 As disclosed herein, an NGR receptor can be a transmembrane receptor. An NGR receptor also can be a molecule that immunoreacts with an anti-CD13 monoclonal antibody and that has aminopeptidase activity. An NGR receptor can have, for example, an amino acid sequence that is substantially

15 similar to the amino acid sequence of CD13/APN (SEQ ID NO:201). Such an NGR receptor can have an amino acid sequence identical to the sequence of CD13/APN (SEQ ID NO:201) or can have one or more modifications, such as deletions, insertions or substitutions, including

20 conservative and non-conservative amino acid substitutions, as long as the receptor remains expressed in angiogenic vasculature and retains specific NGR binding activity. The term "NGR receptor" also is intended to include polypeptides encompassing, for example, modified forms of naturally

25 occurring amino acids such as D-stereoisomers, non-naturally occurring amino acids, amino acid analogues and mimetics, so long as such polypeptides retain functional activity as defined above.

A functional fragment of an NGR receptor also can be

30 useful in the methods of the invention, for example, for identifying tumor homing molecules that home to angiogenic

vasculature of a tumor. As used herein, the term "functional fragment," when used in reference to an NGR receptor, refers to a portion of an NGR receptor that retains some or all binding activity to a homing molecule.

5 Such a functional fragment can be, for example, a domain that binds an NGR motif, such as the extracellular domain of an NGR receptor or an epitope specifically reactive with an antibody. A functional fragment of an NGR receptor useful in identifying a tumor homing molecule can be, for example,

10 the extracellular carboxy-terminal domain of CD13/aminopeptidase N (Look et al., *supra*, 1989).

As used herein, the term "specific binding" means binding that is measurably different from a non-specific interaction. Specific binding can be measured, for example,

15 by determining binding of a molecule compared to binding of a control molecule, which generally is a molecule of similar structure that does not have binding activity, for example, a peptide of similar size that lacks NGR. In this case, specific binding is indicated if the molecule has measurably

20 higher affinity for the NGR receptor than the control molecule. Specificity of binding can be determined, for example, by competition with a control molecule that is known to bind to a target. For example, specific binding of an NGR peptide can be demonstrated by competing for binding

25 with the same NGR peptide or a different peptide containing an NGR motif. In this case, specific binding is indicated if the binding of a molecule is competitively inhibited by the second NGR containing peptide.

The term "specific binding," as used herein, includes

30 both low and high affinity specific binding. Specific

binding can be exhibited, for example, by a low affinity homing molecule having a K_d of at least about 10^{-4} M. For example, if the receptor for a homing molecule has more than one binding site, a homing molecule having low affinity can be useful for targeting angiogenic vasculature. Specific binding also can be exhibited by a high affinity homing molecule, for example, a homing molecule having a K_d of at least about 10^{-7} M, at least about 10^{-8} M, at least about 10^{-9} M, at least about 10^{-10} M, or can have a K_d of at least about 10^{-11} M or 10^{-12} M or greater. Both low and high affinity homing molecules are useful for targeting angiogenic vasculature.

The vasculature within a tumor generally undergoes active angiogenesis, resulting in the continual formation of new blood vessels to support the growing tumor. Such angiogenic blood vessels are distinguishable from mature vasculature in that angiogenic vasculature expresses unique endothelial cell surface markers, including the $\alpha_v\beta_3$ integrin (Brooks, Cell 79:1157-1164 (1994), which is incorporated herein by reference); WO 95/14714, Int. Filing Date November 22, 1994) and receptors for angiogenic growth factors (Mustonen and Alitalo, J. Cell Biol. 129:895-898 (1995); Lappi, Semin. Cancer Biol. 6:279-288 (1995)). Moreover, tumor vasculature is histologically distinguishable from blood vessel in general in that tumor vasculature is fenestrated (Folkman, Nature Med. 1:27-31 (1995); Rak et al., Anticancer Drugs 6:3-18 (1995)). Thus, angiogenic vasculature is a particularly attractive target for targeting a tumor homing molecule. Such a tumor homing molecule can be useful for directing an agent such as a chemotherapeutic drug to a tumor, while reducing the

likelihood the agent will have a toxic effect on normal, healthy organs or tissues (Examples VIII and XV). Moreover, a molecule that homes selectively to angiogenic vasculature also may have use in targeting other types of neovasculature
5 such as that present in inflammatory, regenerating or wounded tissues. As used herein, the term "tumor homing molecule that homes to angiogenic vasculature of a tumor" means a molecule that can bind specifically to a target molecule expressed in angiogenic vasculature of a tumor.
10 Similarly, the term "homing molecule that homes to angiogenic vasculature" means a molecule that can bind specifically to a target molecule expressed in angiogenic vasculature. It is understood that a homing molecule can be a tumor homing molecule.

15 A homing molecule can bind to angiogenic vasculature in a tumor or in non-tumor tissue. A homing molecule that binds to both tumor and non-tumor angiogenic vasculature also can exhibit preferential binding to tumor or non-tumor tissues. For example, a tumor homing peptide such as an NGR
20 peptide can accumulate preferentially in angiogenic vasculature of tumors as compared to non-tumor angiogenic vasculature.

The invention also provides a method of identifying a homing molecule that homes to angiogenic vasculature using
25 substantially purified NGR receptor. The method includes the steps of contacting a substantially purified NGR receptor with one or more molecules and determining specific binding of a molecule to the NGR receptor, where the presence of specific binding identifies the molecule as a
30 homing molecule that homes to angiogenic vasculature.

A method of the invention for identifying a homing molecule also can include the steps of administering an NGR binding molecule *in vivo* and determining binding of the NGR binding molecule to angiogenic vasculature. Thus, the invention provides methods for identifying homing molecules that bind to angiogenic vasculature in non-tumor tissue as well as homing molecules that home to angiogenic vasculature of a tumor. As disclosed herein, a substantially purified NGR receptor can be used to identify homing molecules that home to non-tumor neovascularized tissues of a subject, as well as to identify tumor homing molecules.

A homing molecule that homes to angiogenic vasculature or a tumor homing molecule that homes to angiogenic vasculature of a tumor is identified by screening one or more molecules, for example, a library of molecules. As used herein, the term "library" means a collection of molecules. A library can contain a few or a large number of different molecules, varying from about ten molecules to several billion molecules or more. If desired, a molecule can be linked to a tag, which can facilitate recovery or identification of the molecule. As disclosed herein, a homing molecule that homes to angiogenic vasculature can be identified by *in vitro* screening against a substantially purified NGR receptor.

As used herein, the term "molecule" is used broadly to mean an organic chemical such as a drug; a nucleic acid molecule such as an RNA, a cDNA or an oligonucleotide; a peptide, including a variant or modified peptide or peptide-like molecules, referred to herein as peptidomimetics, which mimic the activity of a peptide; or a protein such as an

antibody or a growth factor receptor or a fragment thereof such as an Fv, single chain Fv(scFv), Fd or Fab fragment of an antibody, which contains a binding domain. For convenience, the term "peptide" is used broadly herein to mean peptides, proteins, fragments of proteins and the like, which can have, for example, a cyclic or linear conformation. A molecule also can be a non-naturally occurring molecule, which does not occur in nature, but is produced as a result of *in vitro* methods, or can be a naturally occurring molecule such as a protein or fragment thereof expressed from a cDNA library or a peptidomimetic.

A molecule to be screened against a substantially purified NGR receptor according to a method of the invention can be a "peptidomimetic," which is used broadly to mean a peptide-like molecule that has the binding activity of a tumor homing peptide, such as a peptidomimetic analog of an NGR peptide. Thus, peptidomimetics, including chemically modified peptides, peptide-like molecules containing non-naturally occurring amino acids, peptoids and the like, and, in particular, peptidomimetics of an NGR containing peptide, can be screened for the ability to specifically bind an NGR receptor, and thus, for activity in homing to angiogenic vasculature (see, for example, "Burger's Medicinal Chemistry and Drug Discovery" 5th ed., vols. 1 to 3 (ed. M.E. Wolff; Wiley Interscience 1995), which is incorporated herein by reference). Peptidomimetics provide various advantages over a peptide, for example, increased stability during passage through the digestive tract and, therefore, are advantageously used for oral administration.

Collections or libraries of peptidomimetics are well known in the art, for example, databases that contain libraries of potential peptidomimetics. For example, the Cambridge Structural Database contains a collection of
5 greater than 300,000 compounds that have known crystal structures (Allen et al., Acta Crystallogr. Section B, 35:2331 (1979)). This structural depository is continually updated as new crystal structures are determined and can be screened for compounds having suitable shapes, for example,
10 the same shape as a tumor homing molecule such as an NGR peptide, as well as potential geometrical and chemical complementarity to a target molecule bound by a tumor homing peptide. Where no crystal structure of a tumor homing peptide or a target molecule, which binds the tumor homing
15 molecule, is available, a structure can be generated using, for example, the program CONCORD (Rusinko et al., J. Chem. Inf. Comput. Sci. 29:251 (1989)). Another database, the Available Chemicals Directory (Molecular Design Limited, Informations Systems; San Leandro CA), contains about
20 100,000 compounds that are commercially available and can be screened to identify a tumor homing molecule or a homing molecule that homes to angiogenic vasculature according to a method of the invention.

Methods for preparing libraries containing diverse
25 populations of various types of molecules such as peptides, peptoids and peptidomimetics are well known in the art and various libraries are commercially available (see, for example, Ecker and Crooke, Biotechnology 13:351-360 (1995), and Blondelle et al., Trends Anal. Chem. 14:83-92 (1995),
30 and the references cited therein, each of which is incorporated herein by reference; see, also, Goodman and Ro,

Peptidomimetics for Drug Design, in "Burger's Medicinal Chemistry and Drug Discovery" Vol. 1 (ed. M.E. Wolff; John Wiley & Sons 1995), pages 803-861, and Gordon et al., J. Med. Chem. 37:1385-1401 (1994), each of which is
5 incorporated herein by reference). Where a molecule is a peptide, protein or fragment thereof, the molecule can be produced *in vitro* directly or can be expressed from a nucleic acid, which can be produced *in vitro*. Methods of synthetic peptide and nucleic acid chemistry are well known
10 in the art.

A library of molecules also can be produced, for example, by constructing a cDNA expression library from mRNA collected from a cell, tissue, organ or organism of interest. Methods for producing such libraries are well
15 known in the art (see, for example, Sambrook et al., Molecular Cloning: A laboratory manual (Cold Spring Harbor Laboratory Press 1989), which is incorporated herein by reference). Preferably, a peptide encoded by the cDNA is expressed on the surface of a cell or a virus containing the
20 cDNA. For example, cDNA can be cloned into a phage vector such as fuse 5 (Example I), wherein, upon expression, the encoded peptide is expressed as a fusion protein on the surface of the phage.

In addition, a library of molecules can comprise a
25 library of nucleic acid molecules, which can be DNA or RNA or an analog thereof. Nucleic acid molecules that bind, for example, to a cell surface receptor are well known (see, for example, O'Connell et al., Proc. Natl. Acad. Sci., USA 93:5883-5887 (1996); Tuerk and Gold, Science 249:505-510
30 (1990); Gold et al., Ann. Rev. Biochem. 64:763-797 (1995),

each of which is incorporated herein by reference). Thus, a library of nucleic acid molecules can be contacted with a substantially purified NGR receptor to identify a tumor homing molecule or a homing molecule that homes to angiogenic vasculature. If desired, the nucleic acid molecules can be nucleic acid analogs that, for example, are less susceptible to attack by nucleases (see, for example, Jelinek et al., Biochemistry 34:11363-11372 (1995); Latham et al., Nucl. Acids Res. 22:2817-2822 (1994); Tam et al., Nucl. Acids Res. 22:977-986 (1994); Reed et al., Cancer Res. 59:6565-6570 (1990), each of which is incorporated herein by reference).

Particularly useful libraries of molecules to be screened for specific binding to an NGR receptor and, therefore, for activity in homing to angiogenic vasculature, include phage display libraries. Such phage display libraries of molecules include secondary libraries expressing NGR in various contexts, including cyclic phage display peptide libraries such as $X_2\text{CNGRCX}_2$ (SEQ ID NO:222), $CX_2(C/X)\text{NGR}(C/X)X_2C$ (SEQ ID NO:223), and CNGRCX_6 (SEQ ID NO:224) (where "C" is cysteine and "X" is any amino acid; see Example X). A library of molecules to be screened also can be a library of antibodies or antibody fragments such as Fv, single chain Fv or Fab fragments; as disclosed in Example X, such a library can be, for example, a combinatorial scFv library prepared from rabbits immunized with human tumor xenografts. Such antibodies can bind to the same epitope recognized by the anti-CD13 antibodies F23 and MY7, or can bind to a different epitope.

One skilled in the art understands that a molecule that specifically binds a substantially purified NGR receptor can bind and modulate the activity of the NGR receptor, or can be inert with respect to its ability to affect the activity of an NGR receptor. As disclosed herein, for example, an NGR receptor is functionally important in angiogenesis. A molecule that specifically binds a substantially purified NGR receptor can be an agonist or an inhibitor of the receptor and, thus, can enhance or inhibit angiogenesis.

10 An inhibitor of an NGR receptor can be highly specific for the NGR receptor. For example, a specific inhibitor can be an antibody that binds with high specificity to an NGR receptor. The antibody can have the inherent property of inhibiting NGR receptor activity upon binding of the
15 antibody. Alternatively, an antibody that is not inhibitory but binds specifically to an NGR receptor can be conjugated to a drug or target to generate a specific inhibitor. The antibody can be a monoclonal or polyclonal antibody or can be a functional antibody fragment such as a Fv, single chain
20 Fv or Fab fragment.

Accordingly, monoclonal or polyclonal antibodies exhibiting specific binding to an NGR receptor can be generated by methods well known to those skilled in the art (Harlow and Lane, Antibodies: A laboratory manual (Cold
25 Spring Harbor Laboratory Press 1988), which is incorporated herein by reference). Alternatively, libraries of functional antibody fragments, which can bind to an NGR receptor, can also be screened to identify a homing molecule that binds to an NGR receptor. For example, a combinatorial
30 scFv library generated by immunizing with human tumor

xenografts or a substantially purified NGR receptor can be screened for binding to an NGR receptor (see Example X).

In addition to inhibitors that are highly specific for an NGR receptor, inhibitors also can exhibit inhibitory activity to other molecules related but not identical to an NGR receptor. For example, aminopeptidase inhibitors can exhibit activity specific for an aminopeptidase or can exhibit inhibitory activity to several aminopeptidases. A homing molecule that binds to an NGR receptor and is an aminopeptidase inhibitor is particularly useful if the inhibitor exhibits preferential binding to an NGR receptor in a target neovascularized tissue.

Accordingly, libraries of molecules to be screened for activity in specifically binding a substantially purified NGR receptor include structural analogs of natural substrates of aminopeptidase as well as structural analogs of aminopeptidase inhibitors. Such libraries can include structural analogs of substrates such as Ala-PNA; Leu enkephalin; Met enkephalin or tuftsin (Xu et al., Experimental Hematology 25:521-529 (1997), which is incorporated herein by reference). Such libraries also can include structural analogs of aminopeptidase inhibitors, such as actinonin; amastatin; bestatin; 1,10-phenanthroline or o-phenanthroline; or 2,2'-dipyridyl; or analogs of Phe-Leu (Xu et al., *supra*, 1997). Such libraries can additionally include structural analogs of fumagillin (Sin et al., Proc. Natl. Acad. Sci. USA 94:6099-6103 (1997), which is incorporated herein by reference). From the above, it is understood that a molecule that specifically binds a

substantially purified NGR receptor can be a naturally or non-naturally occurring structural analog of Phe-Leu.

In addition to screening phage and DNA libraries as described above, combinatorial chemistry libraries also can
5 be screened *in vitro* using a substantially purified NGR receptor according to a method of the invention. Methods for generating combinatorial libraries are well known in the art as described, for example, in Gordon et al., J. Med. Chem. 37:1385-1401 (1994); Gallop et al., J. Med. Chem.
10 37:1203-1251 (1994); and Wilson and Czarnik, eds., Combinatorial Chemistry John Wiley & Sons, New York (1997), each of which is incorporated herein by reference).

The presence of a tumor homing molecule or a homing molecule that specifically binds an NGR receptor within a
15 library of molecules can be identified using various methods well known in the art. Generally, the compounds in a library can be tested individually, for example, using high throughput screening. If desired, the individual compounds can be tagged to facilitate recovery or identification of
20 the molecule. Such tagged libraries are useful for *in vivo* and *in vitro* screening.

As used herein, the term "tag" means a physical, chemical or biological moiety such as a plastic microbead, an oligonucleotide or a bacteriophage, respectively, that is
25 linked to a molecule of the library. Methods for tagging a molecule are well known in the art (Hermanson, Bioconjugate Techniques (Academic Press 1996), which is incorporated herein by reference).

A specific tag can be particularly useful in the methods of the invention for identifying a tumor homing molecule or a homing molecule that homes to angiogenic vasculature. As used herein, the term "specific tag" means
5 a physical, chemical or biological tag that is linked to a particular molecule in a library and is unique for that particular molecule. A specific tag is particularly useful if it is readily identifiable. A nucleotide sequence that is unique for a particular molecule of a library is an
10 example of a specific tag. For example, the method of synthesizing peptides tagged with a unique nucleotide sequence provides a library of molecules, each containing a specific tag, such that upon determining the nucleotide sequence, the identity of the peptide is known (see Brenner
15 and Lerner, Proc. Natl. Acad. Sci., USA 89:5381-5383 (1992), which is incorporated herein by reference). The use of a nucleotide sequence as a specific tag for a peptide or other type of molecule provides a simple means to identify the presence of the molecule in a sample because an extremely
20 sensitive method such as PCR can be used to determine the nucleotide sequence of the specific tag, thereby identifying the sequence of the molecule linked thereto. Similarly, the nucleic acid sequence encoding a peptide expressed on a phage is another example of a specific tag, since sequencing
25 of the specific tag identifies the amino acid sequence of the expressed peptide.

Identified tumor homing molecules are useful, for example, for targeting a desired moiety such as a drug, a toxin or a detectable label, which can be linked to the
30 molecule, to a tumor. In addition, a tumor homing molecule is useful for identifying the target molecule, to which the

homing molecule binds in the tumor. Once a target molecule has been identified, for example, an NGR receptor as disclosed herein, the target molecule can be used to identify additional tumor homing molecules.

5 The present invention also provides a method of directing a moiety to angiogenic vasculature of a tumor in a subject by administering to the subject a conjugate including a moiety linked to a tumor homing molecule that exhibits specific binding to an NGR receptor, whereby the
10 conjugate is directed to angiogenic vasculature of a tumor. In a method of the invention, the tumor homing molecule can be, for example, a peptide containing the sequence NGR, and, if desired, can be part of a conjugate in which the moiety is a cytotoxic agent, drug or chemotherapeutic agent, for
15 example, doxorubicin. A tumor homing peptide containing the sequence NGR can have, for example, the sequence CNGRCVSGCAGRC (SEQ ID NO:3), NGRAHA (SEQ ID NO:6), CVLNGRMEC (SEQ ID NO:7) or CNGRC (SEQ ID NO:8). In a method of the invention for directing a moiety to angiogenic vasculature
20 of a tumor in a subject, the tumor homing molecule also can be, for example, an aminopeptidase inhibitor such as bestatin, o-phenanthroline, actinonin, amastatin, 2,2'-dipyridyl or fumagillin and can be linked, if desired, to a doxorubicin moiety.

25 The present invention additionally provides a method of inhibiting angiogenesis in a tumor of a subject by administering to the subject a conjugate including a moiety linked to a tumor homing molecule that exhibits specific binding to an NGR receptor, whereby the conjugate is
30 directed to angiogenic vasculature of a tumor. In a method

of the invention, the tumor homing molecule can be, for example, a peptide containing the sequence NGR; and, if desired, can be part of a conjugate in which the moiety is a cytotoxic agent, drug or chemotherapeutic agent, for example, doxorubicin. A tumor homing peptide containing the sequence NGR can have, for example, the sequence CNGRCVSGCAGRC (SEQ ID NO:3), NGRAHA (SEQ ID NO:6), CVLNGRMEC (SEQ ID NO:7) or CNGRC (SEQ ID NO:8). In a method of the invention for directing a moiety to angiogenic vasculature of a tumor in a subject, the tumor homing molecule also can be, for example, an aminopeptidase inhibitor such as bestatin, o-phenanthroline, actinonin, amastatin, 2,2'-dipyridyl or fumagillin and can be linked, if desired, to a doxorubicin moiety.

15 The invention also provides a method of inhibiting angiogenesis in a non-tumor tissue. The method includes administering a conjugate including a moiety linked to a homing molecule that exhibits specific binding to an NGR receptor, whereby the conjugate is directed to angiogenic vasculature of a non-tumor tissue. Inhibiting angiogenesis in a non-tumor tissue is useful, for example, for treating diseases involving neovascularized tissue such as retinal neovascularization in macular degeneration and diabetes and neovascularization in rheumatoid arthritis synovium.

25 As disclosed, tumor homing molecules can be conjugated to moieties such as a drug or toxin in order to target the drug or toxin to a tumor. A tumor homing molecule such as one of the NGR containing peptides CNGRCVSGCAGRC (SEQ ID NO:3), NGRAHA (SEQ ID NO:6), CVLNGRMEC (SEQ ID NO:7), or CNGRC (SEQ ID NO:8) can be used to direct a moiety to

30

angiogenic vasculature. Additional tumor homing molecules that bind to the NGR receptor identified *in vivo* or *in vitro* as described above also can be used to direct a moiety to the angiogenic vasculature of a tumor.

5 In addition to tumor homing molecules that contain NGR, other tumor homing molecules that specifically bind to the NGR receptor are also useful for targeting angiogenic vasculature. As described below, the NGR receptor exhibits aminopeptidase activity, and the aminopeptidase activity can
10 be inhibited by known inhibitors such as bestatin, o-phenanthroline and actinonin. Therefore, in addition to NGR containing peptides, other molecules that bind to the NGR receptor can also function as tumor homing molecules. For example, a molecule that functions as an aminopeptidase
15 inhibitor such as bestatin, o-phenanthroline, actinonin, amastatin, 2,2'-dipyridyl or fumagillin can be used as conjugates with a drug or toxin to home to angiogenic vasculature.

The invention additionally provides a method of
20 directing a moiety to angiogenic vasculature in a subject by administering to the subject a conjugate comprising a moiety linked to a homing molecule that exhibits specific binding to an NGR receptor, where the moiety is directed to angiogenic vasculature. Thus, the invention provides a
25 method of directing a conjugate, for example, of a drug or toxin, to angiogenic vasculature of non-tumor tissue. The targeting of a conjugate to angiogenic vasculature of non-tumor tissue is useful, for example, for treating diseases involving neovascularized tissue such as retinal

neovascularization in macular degeneration and diabetes and neovascularization in rheumatoid arthritis synovium.

A variety of moieties can be directed to angiogenic vasculature in a method of the invention. As used herein, the term "moiety" is used broadly to mean a physical, chemical, or biological material that is linked to a tumor homing molecule for the purpose of being targeted *in vivo* to a tumor or to angiogenic vasculature expressing a target molecule recognized by the tumor homing molecule. In particular, a moiety is a biologically useful moiety such as therapeutic moiety, a diagnostic moiety or a drug delivery vehicle. Thus, a moiety can be a therapeutic agent, for example, a cancer chemotherapeutic agent such as doxorubicin, which, when linked to a tumor homing molecule, provides a conjugate useful for treating a cancer in a subject. In addition, a moiety can be a drug delivery vehicle such as a chambered microdevice, a cell, a liposome or a virus, which can contain an agent such as a drug or a nucleic acid.

A moiety also can be a molecule such as a polypeptide or nucleic acid, to which a tumor homing molecule is grafted for the purpose of directing the polypeptide or nucleic acid to a selected tumor (Smith et al., J. Biol. Chem. 269:32788-32795 (1994); Goldman et al., Cancer Res. 15:1447-1451 (1997), each of which is incorporated herein by reference). For example, a peptide tumor homing molecule can be expressed as a fusion protein with a desired polypeptide such that the peptide targets the grafted polypeptide to a selected tumor. Such a desired polypeptide, which is grafted to the tumor homing peptide, can be a polypeptide involved in initiating a cell death pathway, for example,

caspase 8, thus providing a means to direct caspase 8 to a tumor, where it can induce apoptosis of the tumor cells or of the vasculature supplying the tumor. A tumor homing peptide also can be grafted to a polypeptide expressed by a virus, for example, the adenovirus penton base coat protein, thus providing a means to target a virus to a tumor (Wickham et al., Gene Ther. 2:750-756 (1995); Weitzman et al., In: "Gene Therapy and Vector Systems" 2:17-25 (1997), each of which is incorporated herein by reference; see, also, Example III). Such a grafted virus can contain an exogenous gene useful in a method of gene therapy. Accordingly, the invention provides compositions of matter comprising a tumor homing molecule/moiety conjugate.

A moiety can be a detectable label such a radiolabel or can be a cytotoxic agent, including a toxin such as ricin or a drug such as a chemotherapeutic agent or can be a physical, chemical or biological material such as a liposome, microcapsule, micropump or other chambered microdevice, which can be used, for example, as a drug delivery system. Generally, such microdevices, should be nontoxic and, if desired, biodegradable. Various moieties, including microcapsules, which can contain an agent, and methods for linking a moiety, including a chambered microdevice, to a molecule of the invention are well known in the art and commercially available (see, for example, "Remington's Pharmaceutical Sciences" 18th ed. (Mack Publishing Co. 1990), chapters 89-91; Harlow and Lane, Antibodies: A laboratory manual (Cold Spring Harbor Laboratory Press 1988), each of which is incorporated herein by reference; see, also, Hermanson, *supra*, 1996).

As disclosed herein, a moiety can be, for example, a cancer chemotherapeutic agent linked to a tumor homing molecule to produce a tumor homing molecule/moiety conjugate. Cytotoxic chemotherapy is the basis of the systemic treatment of disseminated malignant tumors. However, a major limitation of the currently used chemotherapeutic agents is that these drugs have the narrowest therapeutic index in all of medicine. As such, the dose of cancer chemotherapeutic agents generally is limited by undesirable toxicity to the patient being treated. Thus, the ability of tumor homing peptides of the invention to target drugs into tumors was examined. As disclosed herein, the linking of a cancer chemotherapeutic agent, doxorubicin, to a tumor homing molecule reduced the systemic toxicity of the doxorubicin and enhanced anti-tumor activity of the agent (see Examples VIII and XV).

A conjugate of the invention is exemplified herein by doxorubicin linked to various tumor homing peptides (see Examples VII and VIII). In view of the exemplified method of linking doxorubicin to various tumor homing peptides and the disclosed efficacy of such conjugates of the invention, the skilled artisan will recognize that various other chemotherapeutic agents also can be linked to a tumor homing molecule to make a conjugate of the invention. Cancer chemotherapeutic agents have been linked to antibodies, for example, for the purpose of targeting the agents to cells such as tumor cells that express the antigen recognized by the antibodies. In addition, in such antibody/drug conjugates, the agent can maintain its therapeutic function and the antibody can maintain its antigen binding specificity. For example, the anthracyclin, doxorubicin,

has been linked to antibodies and the antibody/doxorubicin conjugates have been therapeutically effective in treating tumors (Sivam et al., Cancer Res. 55:2352-2356 (1995); Lau et al., Bioorg. Med. Chem. 3:1299-1304 (1995); Shih et al.,
5 Cancer Immunol. Immunother. 38:92-98 (1994)). Similarly, other anthracyclins, including idarubicin and daunorubicin, have been chemically conjugated to antibodies, which have delivered effective doses of the agents to tumors (Rowland et al., Cancer Immunol. Immunother. 37:195-202 (1993);
10 Aboud-Pirak et al., Biochem. Pharmacol. 38:641-648 (1989)).

In addition to the anthracyclins, alkylating agents such as melphalan and chlorambucil have been linked to antibodies to produce therapeutically effective conjugates (Rowland et al., *supra*, 1994; Smyth et al., Immunol. Cell
15 Biol. 65:315-321 (1987)), as have vinca alkaloids such as vindesine and vinblastine (Aboud-Pirak et al., *supra*, 1989; Starling et al., Bioconj. Chem. 3:315-322 (1992)). Similarly, conjugates of antibodies and antimetabolites such as 5-fluorouracil, 5-fluorouridine and derivatives thereof
20 have been effective in treating tumors (Krauer et al., Cancer Res. 52:132-137 (1992); Henn et al., J. Med. Chem. 36:1570-1579 (1993)). Other chemotherapeutic agents, including cis-platinum (Schechter et al., Int. J. Cancer 48:167-172 (1991)), methotrexate (Shawler et al., J. Biol.
25 Resp. Mod. 7:608-618 (1988); Fitzpatrick and Garnett, Anticancer Drug Des. 10:11-24 (1995)) and mitomycin-C (Dillman et al., Mol. Biother. 1:250-255 (1989)) also are therapeutically effective when administered as conjugates with various different antibodies.

The results obtained using antibody/drug conjugates demonstrate that a chemotherapeutic agent can be linked to an antibody to produce a conjugate that maintains the antigen binding specificity of the antibody and the
5 therapeutic function of the agent. As disclosed herein, a conjugate comprising doxorubicin and a tumor homing peptide maintains the tumor homing specificity of the tumor homing peptide as well as the therapeutic efficacy of the chemotherapeutic agent (see Examples VIII and XV). Such
10 results are remarkable, since, in the doxorubicin/CNGRC (SEQ ID NO:8) conjugate, for example, the doxorubicin component has only a slightly lower molecular weight than the peptide and comprises about 46% of the molecular weight of the conjugate.

15 Since the moiety component of a tumor homing molecule/moiety conjugate can comprise a substantial portion of the conjugate without adversely affecting the ability of the tumor homing molecule to home to a tumor, additional components can be included as part of the conjugate, if
20 desired. For example, in some cases, it can be desirable to utilize an oligopeptide spacer between a tumor homing peptide and the moiety (Fitzpatrick and Garnett, Anticancer Drug Des. 10:1-9 (1995)). In this way, panels of moiety/spacer complexes can be constructed, in which a
25 common spacer is linked to various different moieties. Such panels of moiety/spacer conjugates can facilitate linkage of the moiety to a tumor homing molecule such as a tumor homing peptide of choice.

Doxorubicin is one of the most commonly used cancer
30 chemotherapeutic agents and, particularly, is used for

treating breast cancer (Stewart and Ratain, In: "Cancer: Principles and practice of oncology" 5th ed., chap. 19 (eds. DeVita, Jr., et al.; J.P. Lippincott 1997); Harris et al., In "Cancer: Principles and practice of oncology,"
5 *supra*, 1997). In addition, doxorubicin has anti-angiogenic activity (Folkman, *supra*, 1997; Steiner, In "Angiogenesis: Key principles-Science, technology and medicine," pp. 449-454 (eds. Steiner et al.; Birkhauser Verlag, 1992)), which can contribute to its effectiveness in treating cancer.
10 Thus, treatment of human breast cancer xenografts in mice using doxorubicin was selected as a model for exemplifying the present invention.

As used herein, the term "tumor" means a mass of cells that are characterized, at least in part, by containing
15 angiogenic vasculature. The term "tumor" is used broadly to include the tumor parenchymal cells as well as the supporting stroma, including the angiogenic blood vessels that infiltrate the tumor parenchymal cell mass. Although a tumor generally is a malignant tumor, i.e., a "cancer," a
20 tumor also can be nonmalignant, provided that neovascularization is associated with the tumor. The term "normal" or "nontumor" tissue is used to refer to tissue that is not a "tumor." As disclosed herein, a tumor homing molecule can be identified based on its ability to home a
25 tumor, but not to a corresponding nontumor tissue.

As used herein, the term "corresponding," when used in reference to tumors or tissues or both, means that two or more tumors, or two or more tissues, or a tumor and a tissue are of the same histologic type. The skilled artisan will
30 recognize that the histologic type of a tissue is a function

of the cells comprising the tissue. Thus, the artisan will recognize, for example, that a nontumor tissue corresponding to a breast tumor is normal breast tissue, whereas a nontumor tissue corresponding to a melanoma is skin, which contains melanocytes. Furthermore, for purposes of the invention, it is recognized that a tumor homing molecule can bind specifically to a target molecule expressed by the vasculature in a tumor, which generally contains blood vessels undergoing neovascularization, in which case a tissue corresponding to the tumor would comprise nontumor tissue containing blood vessels that are not undergoing active angiogenesis.

The term "corresponding" also is used herein in reference to the evolutionarily conserved nature of target molecules, which are expressed in a tumor, for example, in a mouse as compared to a human. Thus, reference to the corresponding target molecules in mouse tumor vasculature as compared, for example, to human vasculature, means target molecules having a similar function, particularly the ability to specifically bind a tumor homing molecule.

Further provided herein is a method of imaging the angiogenic vasculature of a tumor or other pathological tissue in a subject by administering to the subject a conjugate having a detectable moiety linked to a molecule that exhibits specific binding to an NGR receptor, whereby the conjugate selectively binds the angiogenic vasculature, and detecting the conjugate. A detectable moiety for imaging angiogenic vasculature can be, for example, a radionuclide. A useful homing molecule for imaging angiogenic vasculature can be, for example, a peptide

containing the sequence NGR, such as a peptide containing the sequence CNGRCVSGCAGRC (SEQ ID NO:3), NGRAHA (SEQ ID NO:6), CVLNGRMEC (SEQ ID NO:7) or CNGRC (SEQ ID NO:8). A tumor homing molecule for imaging angiogenic vasculature
5 also can be, for example, an aminopeptidase inhibitor such as bestatin, o-phenanthroline, actinonin, amastatin, 2,2'-dipyridyl or fumagillin. Methods for coupling detectable moieties to a tumor homing molecule are described below. Thus, the identification of tumor homing molecules that bind
10 an NGR receptor as described herein provides reagents useful for imaging tumors for diagnostic and prognostic purposes.

The invention also provides a substantially purified target molecule, comprising an NGR receptor that binds a peptide comprising the sequence NGR, provided that the NGR
15 receptor does not have the amino acid sequence of CD13/aminopeptidase N (SEQ ID NO:201). Accordingly, an NGR receptor that has one or more modifications of amino acids relative to the sequence of CD13/aminopeptidase (SEQ ID NO:201), for example, deletions, insertions or
20 substitutions, including conservative and non-conservative amino acid substitutions, and that specifically binds the sequence NGR is provided by the invention.

The present invention relates to molecules that selectively home to tumors. For example, the invention
25 provides tumor homing peptides such as the peptides CGRECPRLCQSSC (SEQ ID NO:2) and CNGRCVSGCAGRC (SEQ ID NO:3), which were identified based on their ability to home to a breast carcinoma, and the peptide CLSGSLSC (SEQ ID NO:4), which was identified based on its ability to home to a
30 melanoma. Such tumor homing peptides were identified using

in vivo panning (see United States Patent No. 5,622,699, issued April 22, 1997; Pasqualini and Ruoslahti, Nature 380:364-366 (1996), each of which is incorporated herein by reference).

5 The disclosed tumor homing peptides were identified based on their homing to various particular tumors. For example, *in vivo* panning was performed using a mouse bearing a human breast carcinoma xenograft and peptides that homed to the breast tumor were identified. However, as disclosed
10 herein, such tumor homing peptides generally homed to other types of tumors, including a mouse melanoma and a human Kaposi's sarcoma. Thus, while the tumor homing peptide CNGRCVSGCAGRC (SEQ ID NO:3) was identified by its ability to home *in vivo* to a breast tumor, this peptide also homed *in*
15 *vivo* to a melanoma and to a Kaposi's sarcoma, but not to nontumor tissues.

 Similarly, the tumor homing peptide CLSGSLSC (SEQ ID NO:4) was identified based on its homing to melanoma. However, further examination of this peptide revealed that
20 it also homed to a breast tumor and to Kaposi's sarcoma. Immunohistological analysis revealed that such tumor homing peptides initially are associated with the vasculature of the various tumors, although at later time the molecules are associated with tumor parenchymal cells. Thus, the general
25 tumor homing ability of a tumor homing molecule of the invention is due, at least in part, to the ability of the tumor homing molecules to target angiogenic vasculature associated with a tumor. These results indicate that
specific target molecules are expressed by the cells
30 comprising the vasculature in a tumor as compared to the

cell surface molecule expressed by vasculature in nontumor tissues. Using methods as disclosed herein, the artisan readily can determine whether a tumor homing molecule homes generally to the angiogenic vasculature associated with a tumor or homes specifically to a particular type of tumor cell.

Identified tumor homing molecules are useful, for example, for targeting a desired moiety such as a drug, a toxin or a detectable label, which can be linked to the molecule, to a tumor. Thus, the invention provides tumor homing molecule/moiety conjugates, which are useful for targeting the moiety to a tumor. Accordingly, the invention also provides methods of targeting a moiety to a tumor and, therefore, methods of reducing the severity of a tumor and of treating a subject having a cancer (see Examples VIII and XV). In addition, a tumor homing molecule is useful for identifying the target molecule, to which the homing molecule binds in the tumor.

Methods for identifying a tumor homing molecule within a library of molecules have been described hereinabove. Generally, the presence of a tumor homing molecule in a tumor is identified based on one or more characteristics common to the molecules present in the library, then the structure of a particular tumor homing molecule is identified. For example, a highly sensitive detection method such as mass spectrometry, either alone or in combination with a method such as gas chromatography, can be used to identify tumor homing molecules in a tumor. Thus, where a library comprises diverse molecules based generally on the structure of an organic molecule such as a drug, a

tumor homing molecule can be identified by determining the presence of a parent peak for the particular molecule.

If desired, the tumor can be collected, then processed using a method such as HPLC, which can provide a fraction
5 enriched in molecules having a defined range of molecular weights or polar or nonpolar characteristics or the like, depending, for example, on the general characteristics of the molecules comprising the library. Conditions for HPLC will depend on the chemistry of the particular molecule and
10 are well known to those skilled in the art. Similarly, methods for bulk removal of potentially interfering cellular materials such as DNA, RNA, proteins, lipids or carbohydrates are well known in the art, as are methods for enriching a fraction containing an organic molecule using,
15 for example, methods of selective extraction. Where a library comprises a population of diverse organic chemical molecules, each linked to a specific oligonucleotide tag, such that the specific molecule can be identified by determining the oligonucleotide sequence using polymerase
20 chain reaction (PCR), genomic DNA can be removed from the sample of the collected tumor in order to reduce the potential for background PCR reactions. In addition, a library can comprise a population of diverse molecules such as organic chemical molecules, each linked to a common,
25 shared tag. Based on the presence and properties of the shared tag, molecules of the library that selectively home to a tumor can be substantially isolated from a sample of the tumor. These and other methods can be useful for enriching a sample of a collected tumor for the particular
30 tumor homing molecule, thereby removing potentially contaminating materials from the collected tumor sample and

increasing the sensitivity of detecting a molecule.

Evidence provided herein indicates that a sufficient number of tumor homing molecules selectively homes to a tumor during *in vivo* panning such that the molecules readily
5 can be identified. For example, various independent phage expressing the same peptide were identified in tumors formed from implanted human breast cancer cells (Table 1), from mouse melanoma cells (Table 2) or from human Kaposi's sarcoma cells (Table 3).

10 Although a substantial fraction of the identified tumor homing molecules have the same structure, the peptide inserts of only a small number of isolated phage were determined. It should be recognized, however, that hundreds of thousands to millions of phage expressing organ homing
15 peptides have been recovered following *in vivo* pannings for organ homing molecules (see, for example, U.S. Patent No. 5,622,699; Pasqualini and Ruoslahti, *supra*, 1996). These results indicate that specific tumor homing molecules will be present in substantial numbers in a tumor following *in*
20 *vivo* homing, thereby increasing the ease with which the molecules can be identified.

Ease of identification of a tumor homing molecule, particularly an untagged molecule, depends on various factors, including the presence of potentially contaminating
25 background cellular material. Thus, where the tumor homing molecule is an untagged peptide, a larger number must home to the tumor in order to identify the specific peptides against the background of cellular protein. In contrast, a much smaller number of an untagged organic chemical homing

molecule such as a drug is identifiable because such molecules normally are absent from or present in only small numbers in the body. In such a case, a highly sensitive method such as mass spectrometry can be used to identify a
5 tumor homing molecule. The skilled artisan will recognize that the method of identifying a molecule will depend, in part, on the chemistry of the particular molecule.

Several rounds of *in vivo* homing can be performed with partial purification of the library molecule by extracting
10 the target tissue between rounds of screening. To ensure the recovery of an adequate number of homing molecules after the final round of screening, material can be pooled from several animals used in an earlier round of screening and injected into a smaller number of animals in the subsequent
15 rounds of screening. Alternatively, a larger animal can be used in the earlier rounds than in the subsequent rounds of screening. The feasibility of screening without intervening steps has been demonstrated with phage.

Where a tumor homing molecule is a nucleic acid or is
20 tagged with a nucleic acid, an assay such as PCR can be particularly useful for identifying the presence of the molecule because, in principle, PCR can detect the presence of a single nucleic acid molecule (see, for example, Erlich, PCR Technology: Principles and Applications for DNA
25 Amplification (Stockton Press 1989), which is incorporated herein by reference). Preliminary studies have demonstrated that, following intravenous injection of 10 ng of an approximately 6000 base pair plasmid into a mouse and 2 minutes in the circulation, the plasmid was detectable by
30 PCR in a sample of lung. These results indicate that

nucleic acids are sufficiently stable when administered into the circulation such that *in vivo* panning can be used to identify nucleic acid molecules that selectively home to a tumor.

5 The molecules of a library can be tagged, which can facilitate recovery or identification of the molecule. As used herein, the term "tag" means a physical, chemical or biological moiety such as a plastic microbead, an oligonucleotide or a bacteriophage, respectively, that is
10 linked to a molecule of the library. Methods for tagging a molecule are well known in the art (Hermanson, *supra* (1996)), which is incorporated herein by reference).

A tag, which can be a shared tag or a specific tag, can be useful for identifying the presence or structure of a
15 tumor homing molecule of a library. As used herein, the term "shared tag" means a physical, chemical or biological moiety that is common to each molecule in a library. Biotin, for example, can be a shared tag that is linked to each molecule in a library. A shared tag can be useful to
20 identify the presence of a molecule of the library in a sample and also can be useful to substantially isolate the molecules from a sample. For example, where the shared tag is biotin, the biotin-tagged molecules in a library can be substantially isolated by binding to streptavidin or their
25 presence can be identified by binding with a labeled streptavidin. Where a library is a phage display library, the phage that express the peptides are another example of a shared tag, since each peptide of the library is linked to a phage. In addition, a peptide such as the hemagglutinin
30 antigen can be a shared tag that is linked to each molecule

in a library, thereby allowing the use of an antibody specific for the hemagglutinin antigen to substantially isolate molecules of the library from a sample of a selected tumor.

5 A shared tag also can be a nucleic acid sequence that can be useful to identify the presence of molecules of the library in a sample or to substantially isolate molecules of a library from a sample. For example, each of the molecules of a library can be linked to the same selected nucleotide
10 sequence, which constitutes the shared tag. An affinity column containing a nucleotide sequence that is complementary to the shared tag then can be used to hybridize molecules of the library containing the shared tag, thus substantially isolating the molecules from a tumor
15 sample. A nucleotide sequence complementary to a portion of the shared nucleotide sequence tag also can be used as a PCR primer such that the presence of molecules containing the shared tag can be identified in a sample by PCR.

 The molecules of a library can be tagged, which can
20 facilitate recovery or identification of the molecule. As used herein, the term "tag" means a physical, chemical or biological moiety such as a plastic microbead, an oligonucleotide or a bacteriophage, respectively, that is linked to a molecule of the library. Methods for tagging a
25 molecule are well known in the art (Hermanson, Bioconjugate Techniques (Academic Press 1996), which is incorporated herein by reference).

A specific tag can be particularly useful in the methods of the invention for identifying a tumor homing

molecule that homes to angiogenic vasculature. As used herein, the term "specific tag" means a physical, chemical or biological tag that is linked to a particular molecule in a library and is unique for that particular molecule. A
5 specific tag is particularly useful if it is readily identifiable. A nucleotide sequence that is unique for a particular molecule of a library is an example of a specific tag. For example, the method of synthesizing peptides tagged with a unique nucleotide sequence provides a library
10 of molecules, each containing a specific tag, such that upon determining the nucleotide sequence, the identity of the peptide is known (see Brenner and Lerner, *supra* (1992), which is incorporated herein by reference). The use of a nucleotide sequence as a specific tag for a peptide or other
15 type of molecule provides a simple means to identify the presence of the molecule in a sample because an extremely sensitive method such as PCR can be used to determine the nucleotide sequence of the specific tag, thereby identifying the sequence of the molecule linked thereto. Similarly, the
20 nucleic acid sequence encoding a peptide expressed on a phage is another example of a specific tag, since sequencing of the specific tag identifies the amino acid sequence of the expressed peptide.

The presence of a shared tag or a specific tag can
25 provide a means to identify or recover a tumor homing molecule of the invention following *in vivo* panning. In addition, the combination of a shared tag and specific tag can be particularly useful for identifying a tumor homing molecule. For example, a library of peptides can be
30 prepared such that each is linked to a specific nucleotide sequence tag (see, for example, Brenner and Lerner, *supra*,

1992), wherein each specific nucleotide sequence tag has incorporated therein a shared tag such as biotin. Upon homing to a tumor, the particular tumor homing peptides can be substantially isolated from a sample of the tumor based
5 on the shared tag and the specific peptides can be identified, for example, by PCR of the specific tag (see Erlich, *supra*, 1989).

A tag also can serve as a support. As used herein, the term "support" means a tag having a defined surface to which
10 a molecule can be attached. In general, a tag useful as a support is a shared tag. For example, a support can be a biological tag such as a virus or virus-like particle such as a bacteriophage ("phage"); a bacterium such as *E. coli*; or a eukaryotic cell such as a yeast, insect or mammalian
15 cell; or can be a physical tag such as a liposome or a microbead, which can be composed of a plastic, agarose, gelatin or other biological or inert material. If desired, a shared tag useful as a support can have linked thereto a specific tag. Thus, the phage display libraries used in the
20 exemplified methods can be considered to consist of the phage, which is a shared tag that also is a support, and the nucleic acid sequence encoding the expressed peptide, the nucleic acid sequence being a specific tag.

In general, a support should have a diameter less than
25 about 10 μm to about 50 μm in its shortest dimension, such that the support can pass relatively unhindered through the capillary beds present in the subject and not occlude circulation. In addition, a support can be nontoxic, so that it does not perturb the normal expression of cell
30 surface molecules or normal physiology of the subject, and

biodegradable, particularly where the subject used for *in vivo* panning is not sacrificed to collect a selected tumor.

Where a molecule is linked to a support, the tagged
5 molecule comprises the molecule attached to the surface of
the support, such that the part of the molecule suspected of
being able to interact with a target molecule in a cell in
the subject is positioned so as to be able to participate in
the interaction. For example, where the tumor homing
10 molecule is suspected of being a ligand for a growth factor
receptor, the binding portion of the molecule attached to a
support is positioned so it can interact with the growth
factor receptor on a cell in the tumor. If desired, an
appropriate spacer molecule can be positioned between the
15 molecule and the support such that the ability of the
potential tumor homing molecule to interact with the target
molecule is not hindered. A spacer molecule also can
contain a reactive group, which provides a convenient and
efficient means of linking a molecule to a support and, if
20 desired, can contain a tag, which can facilitate recovery or
identification of the molecule (see Hermanson, *supra*, 1996).

As exemplified herein, a peptide suspected of being
able to home to a selected tumor such as a breast carcinoma
or a melanoma was expressed as the N-terminus of a fusion
25 protein, wherein the C-terminus consisted of a phage coat
protein. Upon expression of the fusion protein, the
C-terminal coat protein linked the fusion protein to the
surface of a phage such that the N-terminal peptide was in a
position to interact with a target molecule in the tumor.
30 Thus, a molecule having a shared tag was formed by the

linking of a peptide to a phage, wherein the phage provided a biological support, the peptide molecule was linked as a fusion protein, the phage-encoded portion of the fusion protein acted as a spacer molecule, and the nucleic acid
5 encoding the peptide provided a specific tag allowing identification of a tumor homing peptide.

As used herein, the term "in vivo panning," when used in reference to the identification of a tumor homing molecule, means a method of screening a library by
10 administering the library to a subject and identifying a molecule that selectively homes to a tumor in the subject (see U.S. Patent No. 5,622,699). The term "administering to a subject", when used in referring to a library of molecules or a portion of such a library, is used in its broadest
15 sense to mean that the library is delivered to a tumor in the subject, which, generally, is a vertebrate, particularly a mammal such as a human.

A library can be administered to a subject, for example, by injecting the library into the circulation of
20 the subject such that the molecules pass through the tumor; after an appropriate period of time, circulation is terminated by sacrificing the subject or by removing a sample of the tumor (Example I; see, also, U.S. Patent No. 5,622,699; Pasqualini and Ruoslahti, *supra*, 1996).
25 Alternatively, a cannula can be inserted into a blood vessel in the subject, such that the library is administered by perfusion for an appropriate period of time, after which the library can be removed from the circulation through the cannula or the subject can be sacrificed to collect the
30 tumor, or the tumor can be sampled, to terminate

circulation. Similarly, a library can be shunted through one or a few organs, including the tumor, by cannulation of the appropriate blood vessels in the subject. It is recognized that a library also can be administered to an
5 isolated perfused tumor. Such panning in an isolated perfused tumor can be useful to identify molecules that bind to the tumor and, if desired, can be used as an initial screening of a library.

The use of *in vivo* panning to identify tumor homing
10 molecules is exemplified herein by screening a phage peptide display library in tumor-bearing mice and identifying specific peptides that selectively homed to a breast tumor or to a melanoma tumor (Example I). However, phage libraries that display protein receptor molecules,
15 including, for example, an antibody or an antigen binding fragment of an antibody such as an Fv, Fd or Fab fragment; a hormone receptor such as a growth factor receptor; or a cell adhesion receptor such as an integrin or a selectin also can be used to practice the invention. Variants of such
20 molecules can be constructed using well known methods such as random, site-directed or codon based mutagenesis (see Huse, U.S. Patent No. 5,264,563, issued November 23, 1993, which is incorporated herein by reference) and, if desired, peptides can be chemically modified following expression of
25 the phage but prior to administration to the subject. Thus, various types of phage display libraries can be screened by *in vivo* panning.

Phage display technology provides a means for expressing a diverse population of random or selectively
30 randomized peptides. Various methods of phage display and

methods for producing diverse populations of peptides are well known in the art. For example, Ladner et al. (U.S. Patent No. 5,223,409, issued June 29, 1993, which is incorporated herein by reference) describe methods for preparing diverse populations of binding domains on the surface of a phage. In particular, Ladner et al. describe phage vectors useful for producing a phage display library, as well as methods for selecting potential binding domains and producing randomly or selectively mutated binding domains.

Similarly, Smith and Scott (Meth. Enzymol. 217:228-257 (1993); see, also, Scott and Smith, Science 249: 386-390 (1990), each of which is incorporated herein by reference) describe methods of producing phage peptide display libraries, including vectors and methods of diversifying the population of peptides that are expressed (see, also, Huse, WO 91/07141 and WO 91/07149, each of which is incorporated herein by reference; see, also, Example I). Phage display technology can be particularly powerful when used, for example, with a codon based mutagenesis method, which can be used to produce random peptides or randomly or desirably biased peptides (Huse, U.S. Patent No. 5,264,563, *supra*, 1993). These or other well known methods can be used to produce a phage display library, which can be subjected to the *in vivo* panning method of the invention in order to identify a peptide that homes to a tumor.

In addition to screening a phage display library, *in vivo* panning can be used to screen various other types of libraries, including, for example, an RNA or DNA library or a chemical library. If desired, the tumor homing molecule

can be tagged, which can facilitate recovery of the molecule from the tumor or identification of the molecule in the tumor. For example, where a library of organic molecules, each containing a shared tag, is screened, the tag can be a moiety such as biotin, which can be linked directly to the molecule or can be linked to a support containing the molecules. Biotin provides a shared tag useful for recovering the molecule from a selected tumor sample using an avidin or streptavidin affinity matrix. In addition, a molecule or a support containing a molecule can be linked to a hapten such as 4-ethoxy-methylene-2-phenyl-2-oxazoline-5-one (phOx), which can be bound by an anti-phOx antibody linked to a magnetic bead as a means to recover the molecule. Methods for purifying biotin or phOx labeled conjugates are known in the art and the materials for performing these procedures are commercially available (e.g., Invitrogen, La Jolla CA; and Promega Corp., Madison WI). In the case where a phage library is screened, the phage can be recovered using methods as disclosed in Example I.

In vivo panning provides a method for directly identifying molecules that can selectively home to a tumor. As used herein, the term "home" or "selectively home" means that a particular molecule binds relatively specifically to a target molecule present in the tumor following administration to a subject. In general, selective homing is characterized, in part, by detecting at least a two-fold (2x) greater specific binding of the molecule to the tumor as compared to a control organ or tissue.

It should be recognized that, in some cases, a molecule can localize nonspecifically to an organ or tissue containing a tumor. For example, *in vivo* panning of a phage display library can result in high background in organs such as liver and spleen, which contain a marked component of the reticuloendothelial system (RES). Thus, where a tumor is present, for example, in the liver, nonspecific binding of molecules due to uptake by the RES can make identifying a tumor homing molecule more difficult.

10 Selective homing can be distinguished from nonspecific binding, however, by detecting differences in the abilities of different individual phage to home to a tumor. For example, selective homing can be identified by combining a putative tumor homing molecule such as a peptide expressed
15 on a phage with a large excess of non-infective phage or with about a five-fold excess of phage expressing unselected peptides, injecting the mixture into a subject and collecting a sample of the tumor. In the latter case, for example, provided the number of injected phage expressing
20 tumor homing peptide is sufficiently low so as to be nonsaturating for the target molecule, a determination that greater than about 20% of the phage in the tumor express the putative tumor homing molecule is demonstrative evidence that the peptide expressed by the phage is a specific tumor
25 homing molecule. In addition, nonspecific localization can be distinguished from selective homing by performing competition experiments using, for example, phage expressing a putative tumor homing peptide in combination with an excess amount of the "free" peptide (Example V).

In addition, various methods can be used to prevent nonspecific binding of a molecule to an organ containing a component of the RES. For example, a molecule that homes selectively to a tumor present in an organ containing a component of the RES can be obtained by first blocking the RES using, for example, polystyrene latex particles or dextran sulfate (see Kalin et al., Nucl. Med. Biol. 20:171-174 (1993); Illum et al., J. Pharm. Sci. 75:16-22 (1986); Takeya et al., J. Gen. Microbiol. 100:373-379 (1977), each of which is incorporated herein by reference), then administering the library to the subject. For example, pre-administration of dextran sulfate 500 or polystyrene microspheres prior to administration of a test substance has been used to block nonspecific uptake of the test substance by Kupffer cells, which are the RES component of the liver (Illum et al., *supra*, 1986). Similarly, nonspecific uptake of agents by the RES has been blocked using carbon particles or silica (Takeya et al., *supra*, 1977) or a gelatine colloid (Kalin et al., *supra*, 1993). Thus, various agents useful for blocking nonspecific uptake by the RES are known and routinely used.

Nonspecific binding of phage to RES or to other sites also can be prevented by coinjecting, for example, mice with a specific phage display library together with the same phage made noninfective (Smith et al., *supra*, 1990, 1993). In addition, a peptide that homes to tumor in an organ containing an RES component can be identified by preparing a phage display library using phage that exhibit low background binding to the particular organ. For example, Merrill et al. (Proc. Natl. Acad. Sci., USA 93:3188-3192 (1996), which is incorporated herein by reference) selected

lambda-type phage that are not taken up by the RES and, as a result, remain in the circulation for a prolonged period of time. A filamentous phage variant, for example, can be selected using similar methods.

5 Selective homing can be demonstrated by determining the specificity of a tumor homing molecule for the tumor as compared to a control organ or tissue. Selective homing also can be demonstrated by showing that molecules that home to a tumor, as identified by one round of *in vivo* panning,
10 are enriched for tumor homing molecules in a subsequent round of *in vivo* panning. For example, phage expressing peptides that selectively home to a melanoma tumor were isolated by *in vivo* panning, then were subjected to additional rounds of *in vivo* panning. Following a second
15 round of screening, phage recovered from the tumor showed a 3-fold enrichment in homing to the tumor as compared to brain. Phage recovered from the tumor after a third round of screening showed an average of 10-fold enrichment in homing to the tumor as compared to brain. Selective homing
20 also can be demonstrated by showing that molecules that home to a selected tumor, as identified by one round of *in vivo* panning, are enriched for tumor homing molecules in a subsequent round of *in vivo* panning.

 Tumor homing molecules can be identified by *in vivo*
25 panning using, for example, a mouse containing a transplanted tumor. Such a transplanted tumor can be, for example, a human tumor that is transplanted into immunodeficient mice such as nude mice or a murine tumor that is maintained by passage in tissue culture or in mice.
30 Due to the conserved nature of cellular receptors and of

ligands that bind a particular receptor, it is expected that angiogenic vasculature and histologically similar tumor cells in various species can share common cell surface markers useful as target molecules for a tumor homing molecule. Thus, the skilled artisan would recognize that a tumor homing molecule identified using, for example, *in vivo* panning in a mouse having a murine tumor of a defined histological type such as a melanoma also would bind to the corresponding target molecule in a tumor in a human or other species. Similarly, tumors growing in experimental animals require associated neovascularization, just as that required for a tumor growing in a human or other species. Thus, a tumor homing molecule that binds a target molecule present in the vasculature in a tumor grown in a mouse likely also can bind to the corresponding target molecule in the vasculature of a tumor in a human or other mammalian subject. The general ability of a tumor homing molecule identified, for example, by homing to a human breast tumor, also to home to a human Kaposi's sarcoma or to a mouse melanoma indicates that the target molecules are shared by many tumors. Indeed, the results disclosed herein demonstrate that the target molecules are expressed in the neovasculature, which is host tissue (see Examples V and VIII).

25 A tumor homing molecule identified using *in vivo* panning in an experimental animal such as a mouse readily can be examined for the ability to bind to a corresponding tumor in a human patient by demonstrating, for example, that the molecule also can bind specifically to a sample of the tumor obtained from the patient. For example, the RGD-4C (CDCRGDCFC; SEQ ID NO:1) phage and NGR peptides have been

shown to bind to blood vessels in microscopic sections of human tumors, whereas little or no binding occurs in the blood vessels of nontumor tissues. Thus, routine methods can be used to confirm that a tumor homing molecule
5 identified using *in vivo* panning in an experimental animal also can bind the target molecule in a human tumor.

The steps of administering the library to the subject, collecting a selected tumor and identifying the molecules that home to the tumor, comprise a single round of *in vivo*
10 panning. Although not required, one or more additional rounds of *in vivo* panning generally are performed. Where an additional round of *in vivo* panning is performed, the molecules recovered from the tumor in the previous round are administered to a subject, which can be the same subject
15 used in the previous round, where only a part of the tumor was collected.

By performing a second round of *in vivo* panning, the relative binding selectivity of the molecules recovered from the first round can be determined by administering the
20 identified molecules to a subject, collecting the tumor, and determining whether more phage is recovered from the tumor following the second round of screening as compared to those recovered following the first round. Although not required, a control organ or tissue also can be collected and the
25 molecules recovered from the tumor can be compared with those recovered from the control organ. Ideally, no molecules are recovered from a control organ or tissue following a second or subsequent round of *in vivo* panning. Generally, however, a proportion of the molecules also will
30 be present in a control organ or tissue. In this case, the

ratio of molecules in the selected tumor as compared to the control organ (selected:control) can be determined. For example, phage that homed to melanoma following a first round of *in vivo* panning demonstrated a 3x enrichment in
5 homing to the selected tumor as compared to the control organ, brain, following two additional rounds of panning (Example VI).

Additional rounds of *in vivo* panning can be used to determine whether a particular molecule homes only to the
10 selected tumor or can recognize a target on the tumor that also is expressed in one or more normal organs or tissues in a subject or is sufficiently similar to the target molecule on the tumor. It is unlikely that a tumor homing molecule also will home to a corresponding normal tissue because the
15 method of *in vivo* panning selects only those molecules that home to the tumor, which is selected. Where a tumor homing molecule also directs homing to one or more normal organs or tissues in addition to the tumor, the organs or tissues are considered to constitute a family of selected organs or
20 tissues. Using the method of *in vivo* panning, molecules that home to only the selected tumor can be distinguished from molecules that also home to one or more selected organs or tissues. Such identification is expedited by collecting various organs or tissues during subsequent rounds of *in*
25 *vivo* panning.

The term "control organ or tissue" is used to mean an organ or tissue other than the tumor for which the identification of a tumor homing molecule is desired. A control organ or tissue is characterized in that a tumor
30 homing molecule does not selectively home to the control

organ. A control organ or tissue can be collected, for example, to identify nonspecific binding of the molecule or to determine the selectivity of homing of the molecule. In addition, nonspecific binding can be identified by
5 administering, for example, a control molecule, which is known not to home to a tumor but is chemically similar to a potential tumor homing molecule. Alternatively, where the administered molecules are linked to a support, administration of the supports, alone, also can be used to
10 identify nonspecific binding. For example, a phage that expresses the gene III protein, alone, but that does not contain a peptide fusion protein, can be studied by *in vivo* panning to determine the level of nonspecific binding of the phage support.

15 As disclosed herein, specific homing of a tumor homing molecule readily can be identified by examining the selected tumor tissue as compared to a corresponding nontumor tissue, as well as to control organs or tissues. For example, immunohistological analysis can be performed on a tumor
20 tissue and corresponding nontumor tissue using an antibody specific for a phage used to display tumor homing peptides (see Example V). Alternatively, an antibody can be used that is specific for a shared tag that expressed with the peptide, for example, a FLAG epitope or the like, such
25 detection systems being commercially available.

In general, a library of molecules, which contains a diverse population of random or selectively randomized molecules of interest, is prepared, then administered to a subject. At a selected time after administration, the
30 subject is sacrificed and the tumor is collected such that

the molecules present in the tumor can be identified (see Example I). If desired, one or more control organs or tissues or a part of a control organ or tissue can be sampled. For example, mice bearing a breast tumor or a melanoma tumor were injected with a phage peptide display library, then, after about 1 to 5 minutes, the mice were anesthetized, either frozen in liquid nitrogen or, preferably, are perfused through the heart to terminate circulation of the phage, the tumor and one or more control organs were collected from each, phage present in the tumor and the control organs were recovered and peptides that selectively homed to the respective tumors were identified (see Examples I, II and VI).

In the examples provided, the animals were sacrificed to collect the selected tumor and control organ or tissue. It should be recognized, however, that only a part of a tumor need be collected to recover a support containing a molecule that homes to that tumor and, similarly, only part of an organ or tissue need be collected as a control. Thus, a part of a tumor, for example, can be collected by biopsy, such that a molecule such as a peptide expressed by a phage can be administered to the same subject a second time or more, as desired. Where the molecule that is to be administered a second time to the same subject is tagged or linked, for example, to a support, the tag or support should be nontoxic and biodegradable, so as not to interfere with subsequent rounds of screening.

In vitro screening of phage libraries previously has been used to identify peptides that bind to antibodies or to cell surface receptors (Smith and Scott, *supra*, 1993). For

example, *in vitro* screening of phage peptide display libraries has been used to identify novel peptides that specifically bound to integrin adhesion receptors (Koivunen et al., J. Cell Biol. 124:373-380 (1994a), which is
5 incorporated herein by reference) and to the human urokinase receptor (Goodson et al., Proc. Natl. Acad. Sci., USA 91:7129-7133 (1994)). However, such *in vitro* studies provide no insight as to whether a peptide that can
10 specifically bind to a selected receptor *in vitro* also will bind the receptor *in vivo* or whether the binding peptide or the receptor are unique to a specific organ in the body. Furthermore, the *in vitro* methods are performed using defined, well-characterized target molecules in an artificial system. For example, Goodson et al. (*supra*,
15 1994) utilized cells expressing a recombinant urokinase receptor. However, such *in vitro* methods are limited in that they require prior knowledge of the target molecule and yield little if any information regarding *in vivo* utility.

In vitro panning against cells in culture also has been
20 used to identify molecules that can specifically bind to a receptor expressed by the cells (Barry et al., Nature Med. 2:299-305 (1996), which is incorporated herein by reference). However, the cell surface molecules that are expressed by a cell *in vivo* often change when the cell is
25 grown in culture. Thus, *in vitro* panning methods using cells in culture also are limited in that there is no guarantee a molecule that is identified due to its binding to a cell in culture will have the same binding ability *in vivo*. Furthermore, it is not possible using *in vitro*
30 panning to distinguish molecules that home only to the tumor cells used in the screening, but not to other cell types.

In contrast, *in vivo* panning requires no prior knowledge or availability of a target molecule and identifies molecules that bind to cell surface target molecules that are expressed *in vivo*. Also, since the
5 "nontargeted" tissues are present during the screening, the probability of isolating tumor homing molecules that lack specificity of homing is greatly reduced. Furthermore, in obtaining tumor homing molecules by *in vivo* panning, any molecules that may be particularly susceptible to
10 degradation in the circulation *in vivo* due, for example, to a metabolic activity, are not recovered. Thus, *in vivo* panning provides significant advantages over previous methods by identifying molecules that selectively home *in vivo* and the target molecule present in a tumor.

15 Once a target molecule has been identified, however, *in vitro* screening methods are useful for identifying additional tumor homing molecules. For example, the NGR receptor described herein is a target molecule that is useful for identifying additional tumor homing molecules
20 that home to angiogenic vasculature. Methods of *in vitro* screening are well known in the art. For example, an NGR receptor can be contacted with a library of molecules and screened for binding *in vitro*. If desired, the NGR receptor can be immobilized, for example, to a solid support such as
25 a bead or plate. An NGR receptor can be directly bound to the support, through covalent or non-covalent interactions, or can be immobilized indirectly through a molecule that binds to the NGR receptor. For example, an antibody that binds to an NGR receptor can be used to immobilize an NGR
30 receptor (see Example X). The library is contacted with the NGR receptor *in vitro* and screened for binding activity. A

library with tagged molecules are particularly useful for identifying molecules that bind to an NGR receptor.

Once additional molecules that bind to the target molecule are identified, these additional molecules can be
5 tested *in vivo* to determine if the newly identified molecules can bind to the target molecule and home to angiogenic vasculature *in vivo*. For example, a newly identified molecule that binds to an NGR receptor can be further characterized by screening the molecule *in vivo*
10 using the methods described herein and determining if the newly identified molecule can home to angiogenic vasculature. Thus, the identification of a target molecule such as an NGR receptor can be advantageously used to identify molecules that bind to an NGR receptor and home to
15 angiogenic vasculature.

Although mechanisms by which the disclosed method of *in vivo* panning works have not been fully defined, one possibility is that a molecule such as a peptide expressed on a phage recognizes and binds to a target molecule present
20 on endothelial cells lining the blood vessels in a tumor. Evidence indicates, for example, that the vascular tissues in various organs differ from one another and that such differences can be involved in regulating cellular trafficking in the body. For example, lymphocytes home to
25 lymph nodes or other lymphoid tissues due, in part, to the expression of specific "address" molecules by the endothelial cells in those tissues (Salmi et al., Proc. Natl. Acad. Sci., USA 89:11436-11440 (1992); Springer, Cell 76:301-314 (1994)). Similarly, various leukocytes can
30 recognize sites of inflammation due, in part, to the

expression of endothelial cell markers induced by inflammatory signals (see Butcher and Picker, Science 272:60-66 (1996); Springer, *supra*, 1994). Thus, endothelial cell markers provide a potential target for directing, for example, a drug, which can be linked to a tumor homing molecule, to a tumor in a subject.

In some cases, the metastasis of cancer cells to specific organs also can be due to recognition by the tumor cell of an organ specific marker, including organ specific endothelial cell markers (Fidler and Hart, Science 217:998-1003 (1982)). The pattern of metastasis of many cancers can be explained by assuming that circulating tumor cells are preferentially trapped in the first vascular bed encountered. Thus, the lungs and the liver are the most frequent sites of cancer metastasis. However, some cancers show patterns of metastasis that are not explained by circulatory routing. Metastasis of such cancers may be due to the presence of selectively expressed address molecules such as endothelial cell surface molecules expressed in the organ to which the cancer metastasizes (see Goetz et al., Int. J. Cancer 65:192-199 (1996); Zhu et al., Proc. Natl. Acad. Sci., USA 88:9568-9572 (1991); Pauli et al., Cancer Metast. Rev. 9:175-189 (1990); Nicolson, Biochim. Biophys. Acta 948:175-224 (1988)). The identification of molecules that bind to such organ-specific endothelial cell markers can provide a means to prevent tumor cell metastasis to the particular organ.

Using *in vivo* panning to a breast carcinoma, a melanoma and a Kaposi's sarcoma, phage expressing various peptides that selectively homed to tumors were identified (see

Tables 1, 2 and 3, respectively). Due to the large size of the phage (900-1000 nm) and the short time the phage were allowed to circulate (3 to 5 min), it is unlikely that a substantial number of phage would have exited the circulatory system, particularly in the brain and kidney. Tissue staining studies indicated that the tumor homing molecules that were identified primarily homed to and bound endothelial cell surface markers, which likely are expressed in an organ-specific manner. These results indicate that *in vivo* panning can be used to identify and analyze endothelial cell specificities. Such an analysis is not possible using endothelial cells in culture because the cultured cells tend to lose their tissue-specific differences (Pauli and Lee, Lab. Invest. 58:379-387 (1988)).

Although the conditions under which the *in vivo* pannings were performed identified tumor homing peptides that generally bind to endothelial cell markers, the specific presence of phage expressing tumor homing peptides also was observed in tumor parenchyma, particularly at later times after administration of the peptides (Example V). These results demonstrate that phage expressing peptides can pass through the blood vessels in the tumor, possibly due to the fenestrated nature of the blood vessels, and indicate that the *in vivo* panning method can be useful for identifying target molecules expressed by tumor cells, as well as target molecules expressed by endothelial cells.

Phage peptide display libraries were constructed essentially as described by Smith and Scott (*supra*, 1993; see, also, Koivunen et al., Biotechnology 13:265-270 (1995); Koivunen et al., Meth. Enzymol. 245:346-369 (1994b), each of

which is incorporated herein by reference).
Oligonucleotides encoding peptides having substantially random amino acid sequences were synthesized based on an "NNK" codon, wherein "N" is A, T, C or G and "K" is G or T.
5 "NNK" encodes 32 triplets, which encode the twenty amino acids and an amber STOP codon (Scott and Smith, *supra*, 1990). In some libraries, at least one codon encoding cysteine also was included in each oligonucleotide so that cyclic peptides could be formed through disulfide linkages
10 (Example I). The oligonucleotides were inserted in frame with the sequence encoding the gene III protein (gIII) in the vector fuse 5 such that a peptide-gIII fusion protein can be expressed. Following expression, the fusion protein is expressed on the surface of the phage containing the
15 vector (Koivunen et al., *supra*, 1994b; Smith and Scott, *supra*, 1993).

Following *in vivo* panning, the phage isolated based on their ability to selectively home to human breast carcinoma, mouse melanoma or human Kaposi's sarcoma tumors displayed
20 only a few different peptide sequences (see Tables 1, 2 and 3, respectively). One of the screenings revealed peptide sequences that contained the arginine-glycine-aspartic acid (RGD) integrin recognition sequence (Ruoslahti, Ann. Rev. Cell Devel. Biol. 12:697 (1996)) in the context of a peptide
25 previously demonstrated to bind selectively to α_v -containing integrins (Koivunen et al., *supra*, 1995; WO 95/14714). The sequences of most of the remaining tumor homing peptides did not reveal any significant similarities with known ligands for endothelial cell receptors. However, one of the tumor
30 homing peptides contained the asparagine-glycine-arginine (NGR) motif, which is a weak integrin binding motif similar

to the motifs present in integrin-binding peptides (Ruoslahti et al., U.S. Patent No. 5,536,814, issued July 16, 1996, which is incorporated herein by reference; see, also, Koivunen et al., *supra*, 1994a). Other screenings
5 have revealed numerous NGR-containing peptides (see Table 1). Despite the weak integrin binding ability of NGR peptides, an integrin receptor may not be the target molecule recognized by the NGR tumor homing peptides exemplified herein (Example VIII). As used herein, the term
10 "integrin" means a heterodimeric cell surface adhesion receptor.

The peptides expressed by the phage that homed to the breast tumor included the peptides CGRECPRLCQSSC (SEQ ID NO:2) and CNGRCVSGCAGRC (SEQ ID NO:3; see Table 1; Example
15 II). Similarly, tumor homing peptides, including the peptides CDCRGDCFC (SEQ ID NO:1) and CGSLVRC (SEQ ID NO:5), were identified from two other phage libraries administered to breast tumor bearing mice (Table 1). Some of these motifs, as well as novel one, also were isolated in the
20 screen with mouse melanoma and human Kaposi's sarcoma (see Tables 2 and 3). These results demonstrated that tumor homing molecules can be identified using *in vivo* panning.

Three main tumor homing motifs emerged. As discussed above, one motif contained the sequence RGD (Ruoslahti,
25 *supra*, 1996) embedded in the peptide structure, CDCRGDCFC (SEQ ID NO:1), which is known to bind selectively to α_v integrins (Koivunen et al., *supra*, 1995; WO 95/14714). Since the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are markers of angiogenic vessels (Brooks et al., *supra*, 1994; Friedlander et al.,
30 Science 270:1500 (1995)), a phage expressing the peptide

CDCRGDCFC (SEQ ID NO:1) (designated RGD-4C) was examined for tumor targeting and, as disclosed herein, homed to tumors in a highly selective manner (see Example III). Furthermore, homing by the RGD-4C (CDCRGDCFC; SEQ ID NO:1) phage was
5 inhibited by coadministration of the free CDCRGDCFC (SEQ ID NO:1) peptide.

Another breast tumor homing peptide had the sequence CNGRCVSGCAGRC (SEQ ID NO:3), which contains the NGR motif previously shown to have weak integrin binding activity
10 (Koivunen et al., J. Biol. Chem. 268:20205-20210 (1993), which is incorporated herein by reference),; Koivunen et al., *supra*, 1994a; WO 95/14714). Since an NGR containing peptide was identified, two additional peptides, the linear peptide, NGRAHA (SEQ ID NO:6), and the cyclic peptide,
15 CVLNGRMEC (SEQ ID NO:7), each of which contains the NGR motif, were examined for tumor homing. Like the phage expressing CNGRCVSGCAGRC (SEQ ID NO:3), phage expressing NGRAHA (SEQ ID NO:6) or CVLNGRMEC (SEQ ID NO:7) homed to the tumors. Furthermore, tumor homing was not dependent on the
20 tumor type or on species, as the phage accumulated selectively in human breast carcinoma, as well as in the tumors of mice bearing a mouse melanoma and mice bearing a human Kaposi's sarcoma xenograft.

The various peptides, including RGD- and NGR-containing
25 peptides, generally were bound to the tumor blood vessels. The minimal cyclic NGR peptide, CNGRC (SEQ ID NO:8), was synthesized based on the CNGRCVSGCAGRC (SEQ ID NO:3) sequence. When the CNGRC (SEQ ID NO:8) peptide was co-injected with phage expressing either CNGRCVSGCAGRC (SEQ ID
30 NO:3), NGRAHA (SEQ ID NO:6) or CVLNGRMEC (SEQ ID NO:7),

accumulation of the phage in the breast carcinoma xenografts was inhibited. However, the CNGRC (SEQ ID NO:8) peptide did not inhibit the homing of phage expressing the RGD-4C (CDCRGDCFC; SEQ ID NO:1) peptide, even when administered in
5 amounts up to ten times higher than those that inhibited the homing of the NGR phage. In comparison, the RGD-4C (CDCRGDCFC; SEQ ID NO:1) peptide partially inhibited the homing of the NGR phage, although the amount needed was 5 to 10 fold higher than that of the CNGRC peptide (SEQ ID NO:8).
10 These results indicate that NGR peptides and RGD peptides bind to different receptor sites in tumor vasculature.

A third motif, GSL (glycine-serine-leucine), also was identified following *in vivo* panning in mice bearing breast carcinoma, malignant melanoma or Kaposi's sarcoma. Homing
15 of phage expressing the GSL peptide, CGSLVRC (SEQ ID NO:5), was inhibited by coadministration of the free CGSLVRC (SEQ ID NO:5) peptide. Like the RGD and NGR peptides, phage expressing GSL peptides also bound to blood vessels of tumors. In view of the identification of the conserved RGD,
20 NGR and GSL motifs present in tumor homing peptides, as disclosed herein, it will be recognized that peptides containing such motifs can be useful as tumor homing peptides and, in particular, for forming conjugates that can target a moiety such as a cancer chemotherapeutic agent or a
25 diagnostic agent to a tumor.

Various peptide libraries containing up to 13 amino acids were constructed and the NGR peptide, CNGRCVSGCAGRC (SEQ ID NO:3), was obtained as a result of *in vivo* panning against a breast tumor. This NGR peptide, which was

obtained by screening a random peptide library, was a tumor homing peptide (see Example VIII). In addition, when a peptide library was constructed based on the formula CXXXNGRXX (SEQ ID NO:13) or CXXCNGRCX (SEQ ID NO:14), each
5 of which is biased toward NGR sequences, and used for in vivo panning against a breast tumor, numerous NGR peptides were obtained (see Table 1).

These results indicate that a tumor homing peptide of the invention can comprise the amino acid sequence RGD or
10 NGR or GSL. Such tumor peptides can be as small as five amino acids, such as CNGRC (SEQ ID NO:8). Such tumor homing peptides also can be not only at least 13 amino acids in length, which is the largest peptide exemplified herein, but can be up to 20 amino acids, or 30 amino acids, or 50 to
15 100 amino acids in length, as desired. A tumor homing peptide of the invention conveniently is produced by chemical synthesis.

Immunohistochemical analysis was performed by comparing tissue staining for phage allowed to circulate for about
20 four minutes, followed by perfusion through the heart of the mice, or with tissues analyzed 24 hours after phage injection (see Figure 3). At 24 hours following administration, essentially no phage remain in the circulation and, therefore, perfusion is not required
25 (Pasqualini et al., *supra*, 1997). Strong phage staining was observed in tumor vasculature, but not in normal endothelium, in samples examined four minutes after administration of the CNGRCVSGCAGRC (SEQ ID NO:3) phage (Example V; compare Figures 3E, 3G, 3H and 3J). In
30 comparison, staining of the tumor was strong at 24 hours and

appeared to have spread outside the blood vessels into the tumor parenchyma (compare Figures 3A to 3D and 3F (tumor) with Figures 3I and 3K to 3V (nontumor)). The NGRAHA (SEQ ID NO:6) and CVLNGRMEC (SEQ ID NO:7) phage showed similar
5 staining patterns (Example V). In contrast, the control organs and tissues showed little or no immunostaining, confirming the specificity of the NGR motifs for tumor vessels. Spleen and liver, however, captured phage, as expected, since uptake by the reticuloendothelial system is
10 a general property of phage particles, independent of the presence of peptide expression by the phage (Pasqualini et al., *supra*, 1997).

Immunostaining also was observed following administration of phage expressing the GSL motif containing
15 peptide, CLSGSLSC (SEQ ID NO:4), and, like that of the NGR peptides, was localized to the blood vessels, in this case, within a melanoma tumor (see below; see, also, Examples V and VI). Similarly, immunostaining following administration of phage expressing the RGD motif containing peptide, RGD-4C
20 (CDCRGDCFC; SEQ ID NO:1), to breast tumor bearing mice was localized to the blood vessels in the tumor, but was not observed in brain, kidney or various other nontumor tissues (see Examples III and V; see, also, Pasqualini et al., *supra*, 1997). These results demonstrate that the various
25 tumor homing peptides generally home to tumor vasculature.

The general applicability of the *in vivo* panning method for identifying molecules that home to a tumor was examined by injecting mice bearing a syngeneic melanoma with phage expressing a diverse population of peptides (Example VI).
30 The B16 mouse melanoma model was selected for these studies

because the tumors that form are highly vascularized and because the biology of this tumor line has been thoroughly characterized (see Miner et al., Cancer Res. 42:4631-4638 (1982)). Furthermore, because the B16 melanoma cells are of mouse origin, species differences between the host and the tumor cell donor will not affect, for example, the distribution of phage into the tumor as compared to into normal organs. As disclosed herein, *in vivo* panning against B16 melanoma cells revealed tumor homing peptides, including, for example, the GSL moiety containing peptide CLSGSLSC (SEQ ID NO:4; see, also, Table 2) and immunohistochemical staining of the tumor and other organs using an anti-phage antibody demonstrated that the CLSGSLSC (SEQ ID NO:4) expressing phage resulted in immunostaining in the melanoma, but essentially no staining in skin, kidney or other control organs (Example VI). The staining pattern generally followed the blood vessels within the melanoma, but was not strictly confined to the blood vessels.

Although *in vivo* panning was performed in mice, at least the peptides comprising an NGR, RGD or GSL motif also likely can target human vasculature. The NGR phage binds to blood vessels in the transplanted human breast tumor, but not to blood vessels in normal tissues, indicating that this motif can be particularly useful for tumor targeting in patients. The CDCRGDCFC (SEQ ID NO:1) peptide binds to human α_v -integrins (Koivunen et al., *supra*, 1995), which are selectively expressed in tumor blood vessels of human patients (Max et al., Int. J. Cancer 71:320 (1997); Max et al., Int. J. Cancer 72:706 (1997)). Use of a moiety/CDCRGDCFC (SEQ ID NO:1) conjugate to target the moiety to a tumor also provides the additional advantage

that the moiety will be targeted to tumor cells, themselves, because breast carcinoma cells, for example, can express the $\alpha_v\beta_3$ integrin (Pasqualini et al., *supra*, 1997). In fact, many human tumors express this integrin, which may be

5 involved in the progression of certain tumors such as malignant melanomas (Albelda et al., Cancer Res. 50:6757-6764 (1990); Danen et al., Int. J. Cancer 61:491-496 (1995); Felding-Habermann et al., J. Clin. Invest. 89:2018-2022 (1992); Sanders et al., Cold Spring Harb. Symp. Quant. Biol.

10 58:233-240 (1992); Mitjans et al., J. Cell. Sci. 108:3067-3078 (1995)). Unlike the RGD-4C (CDCRGDCFC; SEQ ID NO:1) peptide, the NGR peptides do not appear to bind to MDA-MD-435 breast carcinoma cells. However, NGR peptides were able to deliver a therapeutically effective amount of doxorubicin

15 to breast tumors (Examples VIII and XV), indicating that, even where a tumor homing molecule homes only to tumor vasculature, i.e., not directly to the tumor cells, such vasculature targeting is sufficient to confer the effect of the moiety linked to the molecule.

20 Since the $\alpha_v\beta_3$ integrin is expressed by endothelial cells in angiogenic vasculature, experiments were performed to determine whether tumor vasculature that is undergoing angiogenesis can be targeted *in vivo* using methods as disclosed herein. Phage expressing the peptide, RGD-4C

25 (CDCRGDCFC; SEQ ID NO:1; see, Koivunen et al., *supra*, 1995), which is known to bind to the $\alpha_v\beta_3$ integrin, were injected into mice bearing tumors formed from human breast carcinoma cells, mouse melanoma cells or human Kaposi's sarcoma cells (see Example V). The RGD-4C (CDCRGDCFC; SEQ ID NO:1) phage

30 selectively homed to each of the tumors, whereas such homing did not occur with control phage. For example, in mice

bearing tumors formed by implantation of human breast carcinoma cells, a twenty- to eighty-fold greater number of the RGD-4C (CDCRGDCFC; SEQ ID NO:1) phage, as compared to unselected control phage, accumulated in the tumor.

- 5 Tissue staining for the phage showed accumulation of the RGD-4C (CDCRGDCFC; SEQ ID NO:1) phage in the blood vessels within the tumor, whereas no staining was observed in brain, kidney or other control organs. Specificity of tumor homing by the RGD-4C (CDCRGDCFC; SEQ ID NO:1) phage
- 10 was demonstrated by competition experiments, in which coinjection of the free RGD-4C (CDCRGDCFC; SEQ ID NO:1) peptide greatly reduced tumor homing of the RGD phage, whereas coinjection of a non-RGD-containing control peptide had no effect on homing of the RGD phage (see Example III).
- 15 These results demonstrate that the $\alpha_v\beta_3$ target molecule is expressed on the luminal surface of endothelial cells in a tumor and that a peptide that binds to an α_v -containing integrin can bind selectively to this integrin and, therefore, to vasculature undergoing angiogenesis.
- 20 The results of these studies indicate that tumor homing molecules can be identified by *in vivo* panning and that, in some cases, a tumor homing molecule can home to vascular tissue in the tumor as well as to tumor parenchyma, probably due to the fenestrated nature of the blood vessels
- 25 permitting ready exit of the phage from the circulatory system. Due to the ability of such tumor homing molecules to home to tumors, the molecules are useful for targeting a linked moiety to tumors. Thus, the invention provides conjugates comprising a tumor homing molecule linked to a
- 30 moiety, such conjugates being useful for targeting the

moiety to tumor cells.

The ability of a molecule that homes to a particular tumor to selectively home to another tumor of the same or a similar histologic type can be determined using, for example, human tumors grown in nude mice or mouse tumors grown in syngeneic mice for these experiments. For example, various human breast cancer cell lines, including MDA-MB-435 breast carcinoma (Price et al., Cancer Res. 50:717-721 (1990)), SKBR-1-II and SK-BR-3 (Fogh et al., J. Natl. Cancer Inst. 59:221-226 (1975)), and mouse mammary tumor lines, including EMT6 (Rosen et al., Int. J. Cancer 57:706-714 (1994)) and C3-L5 (Lala and Parhar, Int. J. Cancer 54:677-684 (1993)), are readily available and commonly used as models for human breast cancer. Using such breast tumor models, for example, information relating to the specificity of an identified breast tumor homing molecule for diverse breast tumors can be obtained and molecules that home to a broad range of different breast tumors or provide the most favorable specificity profiles can be identified. In addition, such analyses can yield new information, for example, about tumor stroma, since stromal cell gene expression, like that of endothelial cells, can be modified by the tumor in ways that cannot be reproduced *in vitro*.

Selective homing of a molecule such as a peptide or protein to a tumor can be due to specific recognition by the peptide of a particular cell target molecule such as a cell surface receptor present on a cell in the tumor. Selectivity of homing is dependent on the particular target molecule being expressed on only one or a few different cell types, such that the molecule homes primarily to the tumor.

As discussed above, the identified tumor homing peptides, at least in part, can recognize endothelial cell surface markers in the blood vessels present in the tumors. However, most cell types, particularly cell types that are
5 unique to an organ or tissue, can express unique target molecules. Thus, *in vivo* panning can be used to identify molecules that selectively home to a particular type of tumor cell such as a breast cancer cell and specific homing can be demonstrated by performing the appropriate
10 competition experiments.

Treatment of human breast cancer xenografts in mice using doxorubicin was selected as a model for exemplifying the present invention. CDCRGDCFC (SEQ ID NO:1) and CNGRG (SEQ ID NO:8) were coupled to doxorubicin (Example VII) and
15 the peptide/doxorubicin conjugates were used to treat mice bearing tumors derived from human MDA-MB-435 breast carcinoma cells (Examples VIII and XV). Mice were treated with 5 µg/week of doxorubicin equivalent (i.e., either free doxorubicin or the doxorubicin component of the
20 peptide/doxorubicin conjugate), as compared to the more commonly used 50-200 µg/mouse used in tumor bearing mice (Berger et al., In "The Nude Mouse in Oncology Research" (CRC Press 1991)). The lower dose was selected because it was expected that the conjugate would be more effective than
25 the free drug.

MDA-MB-435 tumor-bearing mice treated with the doxorubicin/RGD-4C (CDCRGDCFC; SEQ ID NO:1) conjugate had significantly smaller tumors, less spread to regional lymph nodes, and fewer pulmonary metastasis than mice treated with
30 free doxorubicin (see Example VIII). All of the mice

treated with the doxorubicin/RGD-4C (CDCRGDCFC; SEQ ID NO:1) conjugate survived beyond the time when all of the mice treated with free doxorubicin had died from widespread disease. In a dose-escalation experiment, the tumor bearing
5 mice were treated with the doxorubicin/RGD-4C (CDCRGDCFC; SEQ ID NO:1) conjugate at 30 µg/mouse every three weeks for three cycles, then were observed, without further treatment, for an extended period of time. The conjugate treated mice all remained alive more than 6 months after the control,
10 doxorubicin treated mice had died (Example VIII). These results indicate that primary tumor growth and metastasis significantly were inhibited in mice treated with the conjugate and that cures may have occurred.

Many of the mice that received doxorubicin/RGD-4C
15 (CDCRGDCFC; SEQ ID NO:1) conjugate presented marked skin ulceration and tumor necrosis; no such signs were observed in mice treated with free doxorubicin or with doxorubicin conjugated to an unrelated peptide (Example VIII). Histopathological analysis disclosed a pronounced
20 destruction of the vasculature in the tumors treated with conjugate as compared to mice treated with free doxorubicin. Furthermore, when tumors were removed from the mice and the tumor cells plated in culture, viability of cells from the tumors of mice receiving the doxorubicin/RGD-4C (CDCRGDCFC;
25 SEQ ID NO:1) conjugate was about 3 fold less than cells from tumors of mice treated with the free doxorubicin (see Example VIII). These results demonstrate that administration to a tumor bearing mouse of a conjugate comprising a chemotherapeutic agent linked to a tumor homing
30 molecule is more efficacious than administration of the agent, alone, in treating a tumor.

Toxicity was determined by administration of 200 µg/doxorubicin equivalent in mice with very large, size matched breast tumors. All of the mice treated with the doxorubicin/RGD-4C (CDCRGDCFC; SEQ ID NO:1) conjugate
5 survived more than a week, while all of the mice treated with free doxorubicin died within 48 hours of the administration of the drug (Example VIII). These results indicate that accumulation of the tumor homing peptide/doxorubicin conjugate in the large tumors can reduce
10 systemic toxicity of the agent.

Similar toxicity and treatment efficacy results were obtained when breast tumor bearing mice were treated using a doxorubicin/CNGRC (SEQ ID NO:8) conjugate. Tumors in the mice treated with the CNGRC (SEQ ID NO:8) conjugate were
15 significantly smaller than in the control groups; the conjugate suppressed tumor growth almost completely. A strong effect on survival also occurred. Free doxorubicin or doxorubicin conjugated to an unrelated peptide, at the dose used, had little if any effect on tumor growth relative
20 to vehicle alone.

Cytotoxic activity of free doxorubicin and the doxorubicin/peptide conjugates was compared *in vitro* using MDA-MB-435 cells. When cells were exposed to free doxorubicin, doxorubicin/RGD-4C (CDCRGDCFC; SEQ ID NO:1) or
25 doxorubicin conjugated to an unrelated peptide for 30 minutes, cell death occurred only in the cultures treated with the doxorubicin/RGD-4C (CDCRGDCFC; SEQ ID NO:1) conjugate. In comparison, cells were killed by all of the treatments after 24 hours of exposure. These results
30 indicate that enhanced cellular uptake of the

doxorubicin/RGD-4C (CDCRGDCFC; SEQ ID NO:1) conjugate occurs.

As disclosed herein, tumor homing molecules of the invention can bind to the endothelial lining of small blood vessels of tumors. The vasculature within tumors is distinct, presumably due to the continual neovascularization, resulting in the formation of new blood vessels required for tumor growth. The distinct properties of the angiogenic neovasculature within tumors are reflected in the presence of specific markers in endothelial cells and pericytes (Folkman, Nature Biotechnol. 15:510 (1997); Risau, FASEB J. 9:926-933 (1995); Brooks et al., *supra*, 1994); these markers likely are being targeted by the tumor homing molecules of the invention.

The ability of a tumor homing molecule to target the blood vessels in a tumor provides substantial advantages over methods of systemic treatment or methods that directly target the tumor cells. For example, tumor cells depend on a vascular supply for survival and the endothelial lining of blood vessels is readily accessible to a circulating probe. Conversely, in order to reach solid tumor cells, a chemotherapeutic agent must overcome potentially long diffusion distances, closely packed tumor cells, and a dense fibrous stroma with a high interstitial pressure that impedes extravasation (Burrows and Thorpe, Pharmacol. Ther. 64:155-174 (1994)).

In addition, where the tumor vasculature is targeted, the killing of all target cells may not be required, since partial denudation of the endothelium can lead to the

formation of an occlusive thrombus halting the blood flow through the entirety of the affected tumor vessel (Burrows and Thorpe, *supra*, 1994). Furthermore, unlike direct tumor targeting, there is an intrinsic amplification mechanism in tumor vasculature targeting. A single capillary loop can supply nutrients to up to 100 tumor cells, each of which is critically dependent on the blood supply (Denekamp, Cancer Metast. Rev. 9:267-282 (1990); Folkman, *supra*, 1997).

Endothelial cells in a tumor also are unlikely to lose a cell surface target receptor or develop a drug resistance phenotype, as can develop through mutation and clonal evolution of tumor cells, because endothelial cells are genetically stable despite their high proliferation rates (Burrows and Thorpe, *supra*, 1994; Folkman, *supra*, 1995; Folkman, *supra*, 1997). In this regard, it has been long recognized by medical oncologists that, while tumors treated with chemotherapeutic agents commonly develop drug resistance, normal tissues such as bone marrow do not develop such resistance. Thus, toxicity to normal tissues such as chemotherapy induced myelosuppression continues to occur during a treatment, even after tumor cells have become drug resistant. Since the endothelial cells in blood vessels supplying a tumor are nontumor cells, it is expected that they will not develop resistance to chemotherapeutic agents, in a manner analogous to bone marrow cells. In fact, drug resistance has not been observed during long term anti-angiogenic therapy in either experimental animals or in clinical trials (Folkman, *supra*, 1997).

Linking of a moiety larger than an agent such as a drug or other organic or biologic molecule to a tumor homing

molecule for the purpose of directing homing of the moiety to the selected tumor is exemplified by expressing an RGD-containing peptide on a phage, wherein the peptide directed homing of the phage to breast tumor vasculature (Example V). These results indicate that a tumor homing molecule of the invention can be linked to other moieties including, for example, a chambered microdevice or a liposome or a cell such as a white blood cell (WBC), which can be a cytotoxic T cell or a killer cell, wherein upon administration of the tumor homing molecule/WBC conjugate, the molecule directs homing of the WBC to the tumor, where the WBC can exert its effector function.

The linking of a moiety to a tumor homing molecule can result in the molecule directing homing of the linked moiety to a tumor. For example, the linking of a brain homing peptide to a RBC directed homing of the RBC to brain (see U.S. Patent No. 5,622,699; Pasqualini and Ruoslahti, *supra*, 1996). This result indicates that a tumor homing molecule of the invention also can be linked to cell type or to a physical, chemical or biological delivery system such as a liposome or other encapsulating device, which can contain an agent such as drug, in order to direct the cell type or the delivery system to a selected tumor. For example, a tumor homing molecule identified by *in vivo* panning can be linked to a white blood cell (WBC) such as a cytotoxic T cell or a killer cell, wherein upon administration of the tumor homing molecule/WBC conjugate, the molecule directs homing of the WBC to the tumor, where the WBC can exert its effector function. Similarly, a tumor homing molecule can be linked to a liposome or to a chambered microdevice comprising, for example, a permeable or semipermeable membrane, wherein an

agent such as a drug to be delivered to a selected tumor is contained within the liposome or microdevice. Such compositions also can be useful, for example, for delivering a nucleic acid molecule to a tumor cells, thereby providing
5 a means for performing *in vivo* targeted gene therapy.

In one embodiment, a tumor homing molecule is linked to a moiety that is detectable external to the subject, thereby providing a composition useful to perform an *in vivo* diagnostic imaging study. For example, *in vivo* imaging
10 using a detectably labeled tumor homing peptide can identify the presence of a tumor in a subject. For such studies, a moiety such as a gamma ray emitting radionuclide, for example, indium-111 or technitium-99, can be linked to the tumor homing molecule and, following administration to a
15 subject, can be detected using a solid scintillation detector. Similarly, a positron emitting radionuclide such as carbon-11 or a paramagnetic spin label such as carbon-13 can be linked to the molecule and, following administration to a subject, the localization of the moiety/molecule can be
20 detected using positron emission transaxial tomography or magnetic resonance imaging, respectively. Such methods can identify a primary tumor as well as a metastatic lesion, which may not be detectable using other methods. Having identified the presence of a cancer in a subject, in another
25 embodiment of the invention, the tumor homing molecule is linked to a cytotoxic agent such as ricin or a cancer chemotherapeutic agent such as doxorubicin in order to direct the moiety to the tumor or can be linked to a chambered microdevice, which can contain a chemotherapeutic
30 drug or other cytotoxic agent. Use of such a composition provides a means to selectively killing of the tumor, while

substantially sparing normal tissues in a cancer patient and, therefore, the conjugates of the invention provide useful medicaments for diagnosing or treating a cancer patient.

5 The skilled artisan would recognize that various tumor homing molecules can selectively home only to a tumor or can selectively home to a tumor and to a family of selected organs, including, in some cases, the normal tissue counterpart to the tumor. Thus, the artisan would select a
10 tumor homing peptide for administration to a subject based on the procedure being performed. For example, a tumor homing molecule that homes only to a tumor can be useful for directing a therapy to the tumor. In comparison, a tumor homing molecule that selectively homes not only to the
15 tumor, but also to one or more normal organs or tissues, can be used in an imaging method, whereby homing to an organ or tissue other than the tumor provides an internal imaging control. Such an internal control can be useful, for example, for detecting a change in the size of a tumor in
20 response to a treatment, since the normal organ is not expected to change in size and, therefore, can be compared with the tumor size.

Tumor homing peptides, which are identified by in vivo panning, can be synthesized in required quantities
25 using routine methods of solid state peptide synthesis or can be purchased from commercial sources (for example, Anaspec; San Jose CA) and a desired moiety can be linked to the molecule. Several methods useful for linking a moiety to a molecule are known in the art, depending on the
30 particular chemical characteristics of the molecule. For

example, methods of linking haptens to carrier proteins as used routinely in the field of applied immunology (see, for example, Harlow and Lane, *supra*, 1988; Hermanson, *supra*, 1996).

5 It is recognized that, in some cases, a drug can lose cytotoxic efficacy upon conjugation or derivatization depending, for example, on the conjugation procedure or the chemical group utilized (Hurwitz et al., Cancer Res. 35:1175-1181 (1975); Trail et al., Science 261:212-215
10 (1993); Nagy et al., Proc. Natl. Acad. Sci., USA 93:7269-7273 (1996)). Moreover, it is recognized that a phage that yields a tumor homing peptide of the invention displays as many as five of the peptides. Thus, there is a possibility that the affinity of an individual peptide is too low for
15 effective tumor homing and that multivalent, rather than univalent, peptide conjugates must be used. However, as disclosed herein, doxorubicin maintained cytotoxic activity when used as a conjugate with tumor homing peptides (see Examples VIII and XV), thus allaying the potential concerns
20 discussed above.

A moiety such as a therapeutic or diagnostic agent can be conjugated to a tumor homing peptide using, for example, carbodiimide conjugation (Bauminger and Wilchek, Meth. Enzymol. 70:151-159 (1980), which is incorporated herein by
25 reference) (see Example VII). Alternatively, a moiety can be coupled to a homing molecule as described by Nagy et al., Proc. Natl. Acad. Sci. USA 93:7269-7273 (1996); and Nagy et al., Proc. Natl. Acad. Sci. USA 95:1794-1799 (1998), each of which is incorporated herein by reference.

Carbodiimides comprise a group of compounds that have the general formula $R-N=C=N-R'$, where R and R' can be aliphatic or aromatic, and are used for synthesis of peptide bonds. The preparative procedure is simple, relatively
5 fast, and is carried out under mild conditions. Carbodiimide compounds attack carboxylic groups to change them into reactive sites for free amino groups. Carbodiimide conjugation has been used to conjugate a variety of compounds to carriers for the production of
10 antibodies.

The water soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) is particularly useful for conjugating a moiety to a tumor homing peptide and was used to conjugate doxorubicin to tumor homing
15 peptides (Example VII). The conjugation of doxorubicin and a tumor homing peptide requires the presence of an amino group, which is provided by doxorubicin, and a carboxyl group, which is provided by the peptide. EDC coupling of doxorubicin to the CNGRC (SEQ ID NO:8) peptide was performed
20 using a 1:1 molar ratio of the peptide (carboxyl groups) to obtain a doxorubicin/CNGRC (SEQ ID NO:8; see Example VII).

In addition to using carbodiimides for the direct formation of peptide bonds, EDC also can be used to prepare active esters such as N-hydroxysuccinimide (NHS) ester. The
25 NHS ester, which binds only to amino groups, then can be used to induce the formation of an amide bond with the single amino group of the doxorubicin. The use of EDC and NHS in combination is commonly used for conjugation in order to increase yield of conjugate formation (Bauminger and
30 Wilchek, *supra*, 1980).

Other methods for conjugating a moiety to a tumor homing molecule also can be used. For example, sodium periodate oxidation followed by reductive alkylation of appropriate reactants can be used, as can glutaraldehyde crosslinking. However, it is recognized that, regardless of which method of producing a conjugate of the invention is selected, a determination must be made that the tumor homing molecule maintains its targeting ability and that the moiety maintains its relevant function. Methods as disclosed in Examples VIII and XV or otherwise known in the art can confirm the activity of the moiety/tumor homing molecule conjugate.

The yield of moiety/tumor homing molecule conjugate formed is determined using routine methods. For example, HPLC or capillary electrophoresis or other qualitative or quantitative method can be used (see, for example, Liu et al., J. Chromatogr. 735:357-366 (1996); Rose et al., J. Chromatogr. 425:419-412 (1988), each of which is incorporated herein by reference; see, also, Example VII). In particular, the skilled artisan will recognize that the choice of a method for determining yield of a conjugation reaction depends, in part, on the physical and chemical characteristics of the specific moiety and tumor homing molecule. Following conjugation, the reaction products are desalted to remove any free peptide and free drug.

When administered to a subject, the tumor homing molecule/moiety conjugate is administered as a pharmaceutical composition containing, for example, the conjugate and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known in the

art and include, for example, aqueous solutions such as water or physiologically buffered saline or other solvents or vehicles such as glycols, glycerol, oils such as olive oil or injectable organic esters.

5 A pharmaceutically acceptable carrier can contain physiologically acceptable compounds that act, for example, to stabilize or to increase the absorption of the conjugate. Such physiologically acceptable compounds include, for example, carbohydrates, such as glucose, sucrose or
10 dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound,
15 depends, for example, on the route of administration of the composition. The pharmaceutical composition also can contain an agent such as a cancer therapeutic agent.

One skilled in the art would know that a pharmaceutical composition containing a tumor homing molecule can be
20 administered to a subject by various routes including, for example, orally or parenterally, such as intravenously. The composition can be administered by injection or by intubation. The pharmaceutical composition also can be a tumor homing molecule linked to liposomes or other polymer
25 matrices, which can have incorporated therein, for example, a drug such as a chemotherapeutic agent (Gregoriadis, Liposome Technology, Vol. 1 (CRC Press, Boca Raton, FL 1984), which is incorporated herein by reference). Liposomes, for example, which consist of phospholipids or
30 other lipids, are nontoxic, physiologically acceptable and

metabolizable carriers that are relatively simple to make and administer.

For the diagnostic or therapeutic methods disclosed herein, an effective amount of the tumor homing molecule/moiety conjugate must be administered to the subject. As used herein, the term "effective amount" means the amount of the conjugate that produces the desired effect. An effective amount often will depend on the moiety linked to the tumor homing molecule. Thus, a lesser amount of a radiolabeled molecule can be required for imaging as compared to the amount of a drug/molecule conjugate administered for therapeutic purposes. An effective amount of a particular molecule/moiety for a specific purpose can be determined using methods well known to those in the art.

The route of administration of a tumor homing molecule will depend, in part, on the chemical structure of the molecule. Peptides, for example, are not particularly useful when administered orally because they can be degraded in the digestive tract. However, methods for chemically modifying peptides to render them less susceptible to degradation by endogenous proteases or more absorbable through the alimentary tract are well known (see, for example, Blondelle et al., *supra*, 1995; Ecker and Crooke, *supra*, 1995; Goodman and Ro, *supra*, 1995). Such modifications can be performed on peptides identified by *in vivo* panning. In addition, methods for preparing libraries of peptidomimetics, which can contain D-amino acids, other non-naturally occurring amino acids, or chemically modified amino acids; or can be organic molecules that mimic the structure of peptide; or can be peptoids such as vinylogous

peptoids, are known in the art and can be used to identify molecules that home to a tumor and are stable for oral administration.

Tumor homing molecules obtained using the methods disclosed herein also can be useful for identifying a target molecule such as a cell surface receptor or a ligand for a receptor, which is recognized by the tumor homing peptide, or for substantially isolating the target molecule. For example, a tumor homing peptide can be linked to a solid support such as a chromatography matrix. The linked peptide then can be used for affinity chromatography by passing an appropriately processed sample of a tumor over the column in order to bind a particular target molecule. The target molecule, which forms a complex with the tumor homing molecule, then can be eluted from the column and collected in a substantially isolated form. The substantially isolated target molecule then can be characterized using well known methods. A tumor homing peptide also can be linked to a detectable moiety such as a radionuclide, a fluorescent molecule, an enzyme or biotin and can be used, for example, to screen a sample in order to detect the presence of the target molecule in a tumor or to follow the target molecule during various isolation steps.

It follows that, upon identifying the presence of a target molecule in a tumor sample, the skilled artisan readily can obtain the target molecule in a substantially isolated form. For example, the sample containing the target molecule can be passed over a column containing attached thereto the relevant tumor homing molecule, thereby providing a means to obtain the target molecule in

substantially isolated form (see Example X). Thus, the invention further provides a substantially isolated target molecule, which specifically binds a tumor homing molecule and which can be obtained using the methods disclosed
5 herein.

The methods of the present invention were used to identify tumor homing peptides, which can selectively home to various tumors. It should be recognized that cysteine residues were included in some peptides such that
10 cyclization of the peptides could be effected. In fact, the peptides containing at least two cysteine residues cyclize spontaneously. However, such cyclic peptides also can be active when present in a linear form (see, for example, Koivunen et al., *supra*, 1993) and, as disclosed herein, a
15 linear peptide, NGRAHA (SEQ ID NO:6), also was useful as tumor homing molecule (Example VIII; see, also, Table 1). Thus, in some cases one or more cysteine residues in the peptides disclosed herein or otherwise identified as tumor homing peptides can be deleted without significantly
20 affecting the tumor homing activity of the peptide. Methods for determining the necessity of a cysteine residue or of amino acid residues N-terminal or C-terminal to a cysteine residue for tumor homing activity of a peptide of the invention are routine and well known in the art.

25 A tumor homing peptide is useful, for example, for targeting a desired moiety to the selected tumor as discussed above. In addition, a tumor homing peptide can be used to identify the presence of a target molecule in a sample. As used herein, the term "sample" is used in its
30 broadest sense to mean a cell, tissue, organ or portion

thereof, including a tumor, that is isolated from the body. A sample can be, for example, a histologic section or a specimen obtained by biopsy or cells that are placed in or adapted to tissue culture. If desired, a sample can be
5 processed, for example, by homogenization, which can be an initial step for isolating the target molecule to which a tumor homing molecule binds.

A tumor homing peptide such as a breast tumor homing peptide can be used to identify the target molecule
10 expressed in a breast tumor. For example, a breast tumor homing peptide can be attached to a matrix such as a chromatography matrix to produce a peptide affinity matrix. A homogenized sample of a breast tumor can be applied to the peptide-affinity matrix under conditions that allow specific
15 binding of the target molecule to the tumor homing peptide (see, for example, Deutscher, Meth. Enzymol., Guide to Protein Purification (Academic Press, Inc., ed. M.P. Deutscher, 1990), Vol. 182, which is incorporated herein by reference; see, for example, pages 357-379). Unbound and
20 nonspecifically bound material can be removed and the specifically bound breast tumor-derived target molecule can be isolated in substantially purified form. The presence or absence of the target molecule in normal breast tissue also can be determined. Such an analysis can provide insight
25 into methods of treating the tumor.

As disclosed herein, a target molecule, which specifically binds a tumor homing molecule, can be identified by contacting a sample of a tumor with such a tumor homing molecule and identifying a target molecule
30 bound by the tumor homing molecule. In parallel, the tumor

homing molecule is contacted with a sample of a nontumor tissue corresponding to the tumor. The presence of the target molecule in the tumor sample can be identified by determining that the tumor homing molecule does not bind to
5 a component of the corresponding nontumor tissue sample. Thus, the invention provides methods for identifying the presence of a target molecule, which is expressed in a tumor and specifically bound by a tumor homing molecule.

Since numerous tumor homing peptides containing the NGR
10 motif have been identified, for example, a tumor homing peptide comprising an NGR sequence can be used to isolate the NGR receptor. Thus, an NGR tumor homing peptide can be linked to a solid matrix and an appropriately processed sample of a tumor, which specifically binds the NGR peptide,
15 can be passed over the NGR peptide-matrix. The NGR receptor, which is the target molecule for the NGR tumor homing peptide, then can be obtained in a substantially isolated form, as described in Example X. When used in reference to a target molecule, the term "substantially
20 isolated" means that the target molecule comprises at least 30% of the total protein present, although the target molecule can comprise at least 50% of the total protein, or 80% of the total protein, or 90% or 95% of the total protein, or more. A method such as gel electrophoresis and
25 silver staining can be used to determine the relative amount of a target molecule in a sample, following a purification protocol, and, therefore, can be used to identify a substantially isolated target molecule.

The skilled artisan will recognize that a substantially
30 isolated target molecule can be used as an immunogen to

obtain antibodies that specifically bind the target molecule. As used herein, the term "antibody" is used in its broadest sense to include polyclonal and monoclonal antibodies, as well as antigen binding fragments of such antibodies. With regard to an antibody of the invention, which specifically binds a target molecule targeted by a tumor homing molecule, the term "antigen" means the target molecule polypeptide or peptide portion thereof. An antibody or antigen binding fragment of an antibody that binds a target molecule is characterized by having specific binding activity for the target molecule or a peptide portion thereof of at least about $1 \times 10^5 \text{ M}^{-1}$, preferably at least about $1 \times 10^6 \text{ M}^{-1}$, and more preferably at least about $1 \times 10^8 \text{ M}^{-1}$. Thus, Fab, F(ab')_2 , Fd and Fv fragments of the antibody, which retain specific binding activity for a target molecule, which is expressed by angiogenic vasculature, are included within the definition of an antibody.

In addition, the term "antibody" as used herein includes naturally occurring antibodies as well as non-naturally occurring antibodies, including, for example, single chain antibodies, chimeric, bifunctional and humanized antibodies, as well as antigen-binding fragments thereof. Such non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains as described by Huse et al., Science 246:1275-1281 (1989), which is incorporated herein by reference. These and other methods of making, for example, chimeric, humanized, CDR-grafted, single chain, and

bifunctional antibodies are well known to those skilled in the art (Winter and Harris, Immunol. Today 14:243-246 (1993); Ward et al., Nature 341:544-546 (1989); Hilyard et al., Protein Engineering: A practical approach (IRL Press 5 1992); Borrabeck, Antibody Engineering, 2d ed. (Oxford University Press 1995); each of which is incorporated herein by reference; see, also, Harlow and Lane, *supra*, 1988).

Antibodies that specifically bind a target molecule of the invention can be raised using as an immunogen a
10 substantially isolated target molecule, which can be obtained as disclosed herein, or a peptide portion of the target molecule, which can be obtained, for example, by enzymatic degradation of the target molecule and gel purification. A non-immunogenic peptide portion of a target
15 molecule can be made immunogenic by coupling the hapten to a carrier molecule such as bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). Various other carrier molecules and methods for coupling a hapten to a carrier molecule are well known in the art and described, for
20 example, by Harlow and Lane, *supra*, 1988).

Particularly useful antibodies of the invention are those that bind to the tumor homing molecule binding site on the target molecule, such antibodies being readily identifiable by detecting competitive inhibition of binding
25 of the antibody and the particular tumor homing molecule that binds to the target molecule. Conversely, antibodies that bind to an epitope of the target molecule that is not involved in binding the tumor homing molecule also are valuable, since such antibodies, which, themselves, can be

"tumor homing molecules," can be bind to target molecules having another tumor homing molecule bound thereto.

An antibody that specifically binds a target molecule, for example, the NGR receptor, is useful for determining the presence or level of the target molecule in a tissue sample, which can be a lysate or a histological section. The identification of the presence or level of the target molecule in the sample can be made using well known immunoassay and immunohistochemical methods (Harlow and Lane, *supra*, 1988). An antibody specific for a target molecule also can be used to substantially isolate the target molecule from a sample. In addition, an antibody of the invention can be used in a screening assay to identify, for example, peptidomimetics of a tumor homing molecule that bind to the target molecule or as a tool for tumor targeting.

Upon obtaining a target molecule, which, due to the nature of a tumor homing molecule, is expressed in angiogenic vasculature, for example, the angiogenic vasculature in a tumor, the naturally occurring ligand for the target molecule, where it exists, can be identified. Methods for identifying a ligand for such a target molecule, which is akin to an "orphan receptor," are well known in the art and include, for example, screening biological samples to identify the ligand. A convenient screening assay to identify a natural ligand for the target molecule can utilize the ability of a putative natural ligand to competitively inhibit the binding to the target molecule of a tumor homing molecule that specifically binds the target

molecule, for example, the tumor homing peptide used to obtain the substantially isolated target molecule.

A screening assay comprising a competitive binding assay for the target molecule and, for example, the natural
5 ligand for the target molecule or a tumor homing peptide that specifically binds the target molecule, also provides a means to identify peptidomimetics of a tumor homing molecule. As discussed above, such peptidomimetics can provide advantages over tumor homing peptides in that they
10 can be small, relatively stable for storage, conveniently produced in suitable quantities, and capable of being administered orally. A peptidomimetic of a tumor homing peptide can be identified by screening libraries of peptidomimetics in a competitive binding assay as described
15 above.

The disclosed *in vivo* panning method can be used to detect four different kinds of target molecules in tumors. First, because tumor vasculature undergoes active
angiogenesis, target molecules that are characteristic of
20 angiogenic vasculature, in general, or angiogenic tumor vasculature, in particular, can be identified. Second, vascular target molecules that are characteristic of the tissue of origin of the tumor can be identified. Third, target molecules that are expressed in the vasculature of a
25 particular type of tumor can be identified. Fourth, tumor stroma or tumor cell target molecules can be identified due to the fenestrated nature of tumor vasculature, which allows the potential tumor homing molecules to leave the circulation and contact the tumor parenchyma.

As further disclosed herein, some, but not all, tumor homing molecules also can home to angiogenic vasculature that is not contained within a tumor. For example, tumor homing molecules containing either the RGD motif or the GSL motif also homed effectively to retinal neovasculature (Smith et al., Invest. Ophthalmol. Vis. Sci. 35:101-111 (1994), which is incorporated herein by reference), whereas tumor homing peptides containing the NGR motif accumulated to a lesser extent in retinal neovasculature than in tumor vasculature. Thus, the present invention also provides peptides that home to nontumor angiogenic vasculature as well as peptides that home preferentially to tumor vasculature compared to non-tumor neovasculature. Furthermore, these results indicate that tumor vasculature express target molecules that are not substantially expressed by other kinds of angiogenic vasculature. Thus, the present invention provides a means to identify target molecules expressed specifically by angiogenic vasculature present in a tumor, as well as for target molecules expressed by angiogenic vasculature not associated with a tumor. Methods as disclosed herein can be used to distinguish such homing peptides and to isolate the various target molecules.

As an alternative to using a tumor sample to obtain the target molecule, extracts of cultured tumor cells or endothelial cells, depending on which cell type expresses the target molecule, can be used as the starting material in order to enhance the concentration of the target molecule in the sample. It is recognized, however, that the characteristics of such cells can change upon adaptation to tissue culture. Thus, care must be exercised if such a

preselection step is attempted. The presence of the target molecule can be established, for example, by using phage binding and cell attachment assays (see, for example, Barry et al., *supra*, 1996).

5 A cell line expressing a particular target molecule can be identified and surface iodination of the cells can be used to label the target molecule. The cells then can be extracted, for example, with octylglucoside and the extract can be fractionated by affinity chromatography using a tumor
10 homing peptide (see Tables 1 and 2) coupled to a matrix such as SEPHAROSE (see Hermanson, *supra*, 1996). The purified target molecule can be microsequenced and antibodies can be prepared. If desired, oligonucleotide probes can be prepared and used to isolate cDNA clones encoding the target
15 molecule. Alternatively, an anti-target molecule antibody can be used to isolate a cDNA clone from an expression library (see Argraves et al., *J. Cell Biol.* 105:1183-1190 (1987), which is incorporated herein by reference).

As an alternative to isolating the target molecule, a
20 nucleic acid encoding the target molecule can be isolated using a mammalian cell expression cloning system such as the COS cell system. An appropriate library can be prepared, for example, using mRNA from primary tumor cells. The nucleic acids can be cloned into the pcDNAIII vector
25 (Invitrogen), for example. Cells expressing a cDNA for the target molecule can be selected by binding to the tumor homing peptide. Purified phage can be used as the carrier of the peptide and can be attached to magnetic beads coated, for example, with anti-M13 antibodies (Pharmacia Biotech;
30 Piscataway NJ). Cells that bind to the peptide coating can

be recovered using a magnet and the plasmids can be isolated. The recovered plasmid preparations then can be divided into pools and examined in COS cell transfections. The procedure can be repeated until single plasmids are
5 obtained that can confer upon the COS cells the ability to bind the tumor homing peptide.

The following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

10

IN VIVO PANNING

This example demonstrates methods for preparing a phage library and screening the library using *in vivo* panning to identify phage expressing peptides that home to a tumor.

A. Preparation of phage libraries:

15 Phage display libraries were constructed using the fuse 5 vector as described by Koivunen et al. (*supra*, 1995; Koivunen et al., *supra*, 1994b). Libraries encoding peptides designated CX₅C (SEQ ID NO:9), CX₆C (SEQ ID NO:10), CX₇C (SEQ ID NO:11) and CX₃CX₃CX₃C (SEQ ID NO:12) were prepared, where
20 "C" indicates cysteine and "X_N" indicates the given number of individually selected amino acids. These libraries can display cyclic peptides when at least two cysteine residues are present in the peptide. In addition, a library that did not contain defined cysteine residues also was constructed.
25 Such a library results in the production primarily of linear peptides, although cyclic peptides also can occur due to random probability.

A biased library based on the sequence CXXXNGRXX (SEQ ID NO:13) also was constructed. Furthermore, in some cases the CXXXNGRXX (SEQ ID NO:13) library was further biased by the incorporation of cysteine residues flanking the NGR
5 sequence, i.e., CXXCNGRCX (SEQ ID NO:14; see Table 1).

The libraries containing the defined cysteine residues were generated using oligonucleotides constructed such that "C" was encoded by the codon TGT and "X_N" was encoded by NNK, where "N" is equal molar mixtures of A, C, G and T, and
10 where "K" is equal molar mixtures of G and T. Thus, the peptide represented by CX₅C (SEQ ID NO:9) can be represented by an oligonucleotide having the sequence TGT(NNK)₅TGT (SEQ ID NO:14). Oligonucleotides were made double stranded
15 by 3 cycles of PCR amplification, purified and ligated to the nucleic acid encoding the gene III protein in the fuse 5 vector such that, upon expression, the peptide is present as a fusion protein at the N-terminus of the gene III protein.

The vectors were transfected by electroporation into MC1061 cells. Bacteria were cultured for 24 hr in the
20 presence of 20 µg/ml tetracycline, then phage were collected from the supernatant by precipitation twice using polyethylene glycol. Each library contained about 5×10^9 to 5×10^{14} transducing units (TU; individual recombinant phage).

25 B. In vivo panning of phage:

Tumors were transplanted into mice as described in Examples II and III, below. A mixture of phage libraries containing 1×10^9 to 1×10^{14} TU was diluted in 200 µl DMEM

and injected into the tail vein of anesthetized mice (AVERTIN (0.015 ml/g); see U.S. Patent No. 5,622,699; Pasqualini and Ruoslahti, *supra*, 1996). After 1-4 minutes, mice were snap frozen in liquid nitrogen. To recover the phage, carcasses were partially thawed at room temperature for 1 hr, tumors and control organs were collected and weighed, then were ground in 1 ml DMEM-PI (DMEM containing protease inhibitors (PI); phenylmethylsulfonyl fluoride (PMSF; 1 mM), aprotinin (20 µg/ml), leupeptin (1 µg/ml)).

10 Alternatively, following introduction of a library into a mouse, circulation of the library is terminated by perfusion through the heart. Briefly, mice were anesthetized with AVERTIN, then the heart was exposed and a 0.4 mm needle connected through a 0.5 mm cannula to a 10 cc
15 syringe was inserted into the left ventricle. An incision was made on the right atrium and 5 to 10 ml of DMEM was slowly administered, perfusing the whole body over about a 5 to 10 min period. Efficiency of the perfusion was monitored directly by histologic analysis.

20 Tumor and organ samples were washed 3 times with ice cold DMEM-PI containing 1% bovine serum albumin (BSA), then directly incubated with 1 ml K91-kan bacteria for 1 hr. Ten ml NZY medium containing 0.2 µg/ml tetracycline (NZY/tet) was added to the bacterial culture, the mixture was
25 incubated in a 37°C shaker for 1 hr, then 10 µl or 100 µl aliquots were plated in agar plates containing 12.5 µg/ml tetracycline (tet/agar).

Individual colonies containing phage recovered from a tumor were grown for 16 hr in 5 ml NZY/tet. The bacterial

cultures obtained from the individual colonies were pooled and the phage were purified and re-injected into mice as described above for a second round of *in vivo* panning. In general, a third round of panning also was performed. Phage
5 DNA was purified from individual bacterial colonies obtained from the final round of *in vivo* panning and the DNA sequences encoding the peptides expressed by selected phage were determined (see Koivunen et al., *supra*, 1994b).

EXAMPLE II

10 IDENTIFICATION OF TUMOR HOMING PEPTIDES BY IN VIVO PANNING AGAINST A BREAST TUMOR

This example demonstrates that *in vivo* panning can be performed against a breast tumor to identify tumor homing peptides that home to various tumors.

15 Human 435 breast carcinoma cells (Price et al., *supra* (1990)) were inoculated into the mammary fat pad of nude mice. When the tumors attained a diameter of about 1 cm, either a phage targeting experiment was performed, in which phage expressing a specific peptide were administered to the
20 tumor bearing mouse, or *in vivo* panning was performed.

The breast tumor bearing mice were injected with 1×10^9 phage expressing a library of CX₃CX₃CX₃C (SEQ ID NO:12) peptides, where X₃ indicates three groups of independently selected, random amino acids. The phage were
25 allowed to circulate for 4 min, then the mice were anesthetized, snap frozen in liquid nitrogen while under anesthesia, and the tumor was removed. Phage were isolated

from the tumor and subjected to two additional rounds of *in vivo* panning.

Following the third round of panning, phage were quantitated and the peptide sequences expressed by the
5 cloned phage were determined. The cloned phage expressed various different peptides, including those shown in Table 1. Similarly, CX₇C (SEQ ID NO:11) and CX₅C (SEQ ID NO:9) libraries were screened and breast tumor
10 homing peptides were identified (Table 1). These results demonstrate that *in vivo* panning against a breast tumor can identify tumor homing molecules.

TABLE 1

PEPTIDES FROM PHAGE RECOVERED FROM HUMAN BREAST CANCER

5			
	CGRECPRLCQSSC (2*)	CNGRCVSGCAGRC (3)	
	CGEACGGQCALPC (20)	IWSGYGVYW (21)	
	PSCAYMCIT (22)	WESLYFPRE (23)	
10	SKVLYYNWE (24)	CGLMCQGACFDVC (25)	
	CERACRNLCREGC (26)	CPRGCLAVCVSQC (27)	
	CKVCNGRCCG (28)	CEMCNGRCMG (29)	CPLCNGRCAL (30)
	CPTCNGRCVR (31)	CGVCNGRCGL (32)	CEQCNGRCGQ (33)
	CRNCNGRCEG (34)	CVLCNGRCWS (35)	CVTCNGRCRV (36)
15	CTECNGRCQL (37)	CRTCNGRCLE (38)	CETCNGRCVG (39)
	CAVCNGRCGF (40)	CRDLNGRKVM (41)	CSCCNGRCGD (42)
	CWGCNGRCRM (43)	CPLCNGRCAR (44)	CKSCNGRCCLA (45)
	CVPCNGRCHE (46)	CQSCNGRCVR (47)	CRTCNGRCQV (48)
	CVQCNGRCAL (49)	CRCCNGRCSP (50)	CASNNGRVVL (51)
20	CGRNGRCLL (52)	CWLCNGRCGR (53)	CSKCNGRCGH (54)
	CVWCNGRCGL (55)	CIRCNGRCVS (56)	CGECNGRCVE (57)
	CEGVNGRRLR (58)	CLSCNGRCPS (59)	CEVCNGRCAL (60)
	CGSLVRC (5)	GRSQMQI (61)	HHTRFVS (62)
	SKGLRHR (63)	VASVSVA (64)	WRVLA AF (65)
25	KMGPKVW (66)	IFSGSRE (67)	SPGSWTW (68)
	NPRWFWD (69)	GRWYKWA (70)	IKARASP (71)
	SGWCYRC (72)	ALVGLMR (73)	LWAEMTG (74)
	CWSGVDC (75)	DTLRLRI (76)	SKSSGVS (77)
	IVADYQR (78)	VWRTGHL (79)	VVDRFPD (80)
30	LSMFTRP (81)	GLPVKWS (82)	IMYPGWL (83)
	CVMVRDGDC (84)	CVRIRPC (85)	CQLAAVC (86)
	CGVGSSC (87)	CVSGPRC (88)	CGLSDSC (89)
	CGEGHPC (90)	CYTADPC (91)	CELSLISKC (92)
	CPEHRSLVC (93)	CLVVHEAAC (94)	CYVELHC (95)
35	CWRKFYC (96)	CFWPNRC (97)	CYSYFLAC (98)
	CPRGSRC (99)	CRLGIAC (100)	CDDSWKC (101)
	CAQLLQVSC (102)	CYPADPC (103)	CKALSQAC (104)
40	CTDYVRC (105)	CGETMRC (106)	

* - numbers in parentheses indicate SEQ ID NO:.

EXAMPLE III

IN VIVO TARGETING OF A PHAGE EXPRESSING
AN RGD PEPTIDE TO A TUMOR

Human 435 breast carcinoma cells were inoculated into
5 the mammary fat pad of nude mice. When the tumors attained
a diameter of about 1 cm, phage expressing a specific
RGD-containing peptide were administered to the tumor
bearing mouse. Similar results
to those discussed below also were obtained with nude mice
10 bearing tumors formed by implantation of human melanoma
C8161 cells or by implantation of mouse B16 melanoma cells.

1 x 10⁹ phage expressing the RGD-containing peptide,
RGD-4C (CDCRGDCFC; SEQ ID NO:1; see, Koivunen et al., *supra*,
1995) or control (insertless) phage were injected
15 intravenously (iv) into the mice and allowed to circulate
for 4 min. The mice then were snap frozen or perfused
through the heart while under anesthesia, and various
organs, including tumor, brain and kidney, were removed and
the phage present in the organs was quantitated (see U.S.
20 Patent No. 5,622,699; Pasqualini and Ruoslahti, *supra*,
1996).

Approximately 2-3 times more phage expressing the RGD-
4C (CDCRGDCFC; SEQ ID NO:1) peptide were detected in the
breast tumor as compared to brain and kidney, indicating the
25 CDCRGDCFC (SEQ ID NO:1; RGD-4C phage) peptide resulted in
selective homing of the phage to the breast tumor. In a
parallel study, unselected phage, which express various,
diverse peptides, were injected into tumor-bearing mice and
various organs were examined for the presence of phage. Far

more phage were present in kidney and, to a lesser extent, brain, as compared to the tumor. Thus, the 80-fold more RGD-expressing phage than unselected phage concentrated in the tumor. These results indicate that phage expressing the RGD-containing peptide home to a tumor, possibly due to the expression of the $\alpha_v\beta_3$ integrin on blood vessels forming in the tumor.

Specificity of the breast tumor homing peptide was demonstrated by competition experiments, in which coinjection of 500 μ g free peptide, ACDCRGDCFCG (SEQ ID NO:16; see Pasqualini et al., *supra*, 1997) with the phage expressing the tumor homing peptide reduced the amount of phage in the tumor by about tenfold, whereas coinjection with the inactive control peptide, GRGESP (SEQ ID NO:17) essentially had no effect. These results demonstrate that phage displaying a peptide that can bind to an integrin expressed on angiogenic vasculature can selectively home *in vivo* to an organ or tissue such as a tumor containing such vasculature.

20

EXAMPLE IV

IN VIVO TARGETING OF PHAGE EXPRESSING TUMOR HOMING PEPTIDES

This example describes *in vivo* targeting of phage expressing peptides that home to tumors.

One of the peptides that accumulated in tumors was derived from a library with the structure CX,CX,CX,C (Arap et al., *Science* 279:377-380 (1998), which is incorporated herein by reference). This peptide, CNGRCVSGCAGRC (SEQ ID NO:3), contained the sequence NGR (asparagine-glycine-

arginine), which has been identified previously as a weak cell adhesion motif (Koivunen et al., J. Biol. Chem. 268:20205-20210 (1993); Healy et al., Biochem. 34:3948-3955 (1995), each of which is incorporated herein by reference).

5 Two other peptides that contain the NGR motif, but are otherwise entirely different from the CNGRCVSGCAGRC (SEQ ID NO:3) peptide, were tested for the ability to home to various tumors *in vivo*. One of them is a linear peptide, NGRAHA (SEQ ID NO:6) (Koivunen et al. *supra* (1993) and the

10 other a cyclic one, CVLNGRMEC.

Phage displaying tumor homing peptides were recovered from breast carcinoma xenografts. Briefly, phage were injected at 10^9 transducing units (TU) into the tail vein of mice bearing size-matched MDA-MB-435 derived tumors (~1000

15 mm³) and recovered after perfusion. The mean number of phage recovered from the tumor or control tissue (brain) and the standard error of the mean (SEM) from triplicate platings are shown in Figure 1. Figure 1A, left panel, shows recovery of CNGRCVSGCAGRC (SEQ ID NO:3) phage from

20 tumor (filled bars) and brain (striped bars). The minimal cyclic NGR peptide from the CNGRCVSGCAGRC (SEQ ID NO:3) phage (CNGRC) was synthesized, and, when 500 µg of CNGRC (SEQ ID NO:8) peptide was co-injected with the phage, the peptide inhibited the accumulation of CNGRCVSGCAGRC (SEQ ID

25 NO:3) phage as shown in Figure 1B. The CNGRC (SEQ ID NO:8) peptide also inhibited the accumulation of the two other NGR-displaying phage in breast carcinoma xenografts.

Figure 1A, middle panel, shows recovery of CGSLVRC (SEQ ID NO:5) phage and inhibition of tumor homing by the soluble

30 peptide CGSLVRC (SEQ ID NO:5) (500 µg). Figure 1A, right panel, shows recovery of RGD-4C (CDCRGDCFC; SEQ ID NO:1)

phage (positive control) and unselected phage library mix (negative control).

In Figure 1B, left panel, increasing amounts of the RGD-4C (CDCRGDCFC; SEQ ID NO:1) soluble peptide were
5 injected with the CNGRCSVSGCAGRC (SEQ ID NO:3) phage. In Figure 1B, right panel, increasing amounts of the CNGR (SEQ ID NO:8) soluble peptide were injected with the RGD-4C (CDCRGDCFC; SEQ ID NO:1) phage. Inhibition of the CNGRCSVSGCAGRC (SEQ ID NO:3) phage homing by the CNGR (SEQ
10 ID NO:8) peptide is shown in Figure 1A. Inhibition of the RGD-4C (CDCRGDCFC; SEQ ID NO:1) phage by the RGD-4C (CDCRGDCFC; SEQ ID NO:1) peptide is described above.

As shown in Figure 1, the CNGRCSVSGCAGRC (SEQ ID NO:3) phage homed specifically to the xenografted human breast
15 carcinomas. Similar results were obtained with the other NGR-displaying phage. The tumor homing was not dependent on the tumor type or on the species of the tumor: the phage accumulated selectively in the human breast carcinoma used in the selection as shown in Figure 1A, as well as in a
20 human Kaposi's sarcoma and in a mouse melanoma (data not shown).

As shown in Figure 1B, left panel, the RGD-4C (CDCRGDCFC; SEQ ID NO:1) phage homes selectively to the breast cancer tumor, and its homing is readily inhibited by
25 free RGD-4C peptide (see above). However, the tumor homing of RGD-4C phage was not inhibited by the CNGR peptide (Figure 1B, left panel), even when the peptide was used in amounts higher than those that inhibited the homing of the NGR phage (Figure 1A, left panel). As shown in Figure 1B,

right panel, the tumor homing of the NGR phage was partially inhibited by the RGD-4C (CDCRGDCFC; SEQ ID NO:1) peptide, but this peptide was 5-10 times less potent than the CNGRC peptide. An unrelated cyclic peptide, GACVFSIAHECGA (SEQ ID NO:19), had no effect in the tumor homing ability of either phage (data not shown). In sum, these results indicate that the two peptides displaying RGD and NGR bind to different receptor sites in tumor vasculature, although the RGD and NGR receptor sites can be related.

10 To further characterize and quantitate the tumor homing ability of phage expressing peptides, CNGRC (SEQ ID NO:8), RGD-4C (CDCRGDCFC; SEQ ID NO:1), and control phage (insertless fd-amp and fd-tet) were injected at 10^9 transducing units (TU) into the tail vein of mice bearing
15 size-matched, MDA-MB-435-derived tumors ($\sim 1000 \text{ mm}^3$) and recovered from tumors and brain tissue after perfusion. An insertless phage with a different selective marker (ampicillin instead of tetracycline) was co-injected with the NGR phage at the same input to assess specificity within
20 the same tumor-bearing animal. The ratios were calculated using the number of co-injected control ampicillin-resistant phage recovered from the same tissues. The phage were quantitated from triplicate platings on tetracycline (the homing phage) or ampicillin (control phage) selective
25 plates. Various additional control phage were injected separately into tumor-bearing mice. These controls included: fd-tet phage without insert; unselected phage library mixtures; phage selected and shown to home to other normal vascular beds; and phage displaying peptides that
30 were unrelated to NGR. The RGD-4C (CDCRGDCFC; SEQ ID NO:1) phage served as a positive control.

110

Over 10-fold more NGR phage than control ampicillin-resistant phage accumulated in the tumor in the co-injection experiments (Figure 2). The RGD-4C (CDCRGDCFC; SEQ ID NO:1) phage also bound selectively to the tumors. In contrast, 5 more ampicillin phage than NGR phage were recovered from brain (Figure 2) and other control organs tested. Moreover, co-injection of the NGR-phage with a 10-fold excess of phage particles engineered to be non-infective did not affect tumor homing. The inclusion of excess non-infective phage 10 particles did decrease the trapping of phage in organs containing reticuloendothelial tissue such as the spleen and the liver.

EXAMPLE V

IMMUNOHISTOCHEMICAL ANALYSIS OF TUMOR HOMING PEPTIDES

15 This example provides a method of identifying the localization of tumor homing molecules by immunohistochemical examination.

Localization of phage expressing a tumor homing peptide was identified by immunochemical methods in histologic 20 sections obtained either 5 min or 24 hr after administration of phage expressing a tumor homing peptide ("peptide-phage") to a tumor bearing mouse (Figure 3). For samples obtained 5 min following administration of the peptide-phage, mice were perfused with DMEM and various organs, including the tumor, 25 were removed and fixed in Bouin's solution. For samples obtained at 24 hr, no peptide-phage remains in the circulation and, therefore, perfusion was not required. Histologic sections were prepared and reacted with anti-M13 (phage) antibodies (Pharmacia Biotech; see U.S. Patent

No. 5,622,699; Pasqualini and Ruoslahti, *supra*, 1996). Visualization of the bound anti-M13 antibody was performed using a peroxidase-conjugated second antibody (Sigma; St. Louis MO) according to the manufacturer's instructions.

5 As discussed in Example III, phage expressing the tumor homing peptide, RGD-4C (CDCRGDCFC; SEQ ID NO:1; also designated "RGD phage"), were administered intravenously to mice bearing the breast tumor. In addition, the RGD phage were administered to mice bearing a mouse melanoma or a
10 human Kaposi's sarcoma. Circulation of the phage was terminated and mice were sacrificed as described above and samples of the tumor and of skin adjacent to the tumor, brain, kidney, lung and liver were collected. Immunohistochemical staining for the phage showed
15 accumulation of the RGD phage in the blood vessels present in the breast tumor as well as in the melanoma and the Kaposi's sarcoma, whereas little or no staining was observed in the control organs.

Similar experiments were performed using phage
20 expressing the tumor homing peptide, CNGRCSVSGCAGRC (SEQ ID NO:3; "NGR phage"), which was identified by *in vivo* panning against a tumor formed by the MDA-MB-435 breast carcinoma. In these experiments, NGR phage or control phage, which do not express a peptide, were administered to mice bearing
25 tumors formed by the MDA-MB-435 breast carcinoma or by a human SLK Kaposi's sarcoma xenograft, then the mice were sacrificed as described above and tumors were collected as well as control organs, including brain, lymph node, kidney, pancreas, uterus, mammary fat pad, lung, intestine, skin,
30 skeletal muscle, heart and epithelium of the renal calices,

bladder and ureter (see Figure 3). Histological samples were prepared and examined by immunostaining as described above.

In samples obtained from mice sacrificed 4 min after administration of the NGR phage, immunostaining of the vasculature of both the breast tumor (Figure 3E) and the Kaposi's sarcoma (Figure 3H) was observed. Very little or no staining was observed in the endothelium of these tumors in mice administered an insertless control phage (Figures 3G and 3J, respectively). In the samples obtained from mice sacrificed 24 hr after administration of the NGR phage, staining of the tumor samples appeared to have spread outside of the vessels, into the breast tumor parenchyma (Figures 3B and 3F) and the Kaposi's sarcoma parenchyma (Figures 3D and 3I). Again, little or no staining was observed in samples prepared from these tumors in mice administered the insertless control phage (Figures 3A and 3C, respectively). In addition, little or no staining was observed in various control organs in mice administered the NGR phage (Figures 3K to 3V).

In other experiments, similar results were obtained following administration of phage expressing the NGR tumor homing peptides, NGRAHA (SEQ ID NO:6) or CVLNGRMEC (SEQ ID NO:7), to tumor bearing mice. Also, as discussed below, similar results were obtained using phage expressing the GSL tumor homing peptide, CLSGSLSC (SEQ ID NO:4), which was identified by *in vivo* panning of a melanoma (see Example VI, below).

These results demonstrate that tumor homing peptides selectively home to tumors, particularly to the vasculature in the tumors and that tumor homing peptides identified, for example, by *in vivo* panning against a breast carcinoma also
5 selectively home to other tumors, including Kaposi's sarcoma and melanoma. In addition, these results demonstrate that immunohistochemical analysis provides a convenient assay for identifying the localization of phage expressing tumor homing peptides.

10

EXAMPLE VI

IDENTIFICATION OF TUMOR HOMING PEPTIDES BY *IN VIVO* PANNING AGAINST A MELANOMA TUMOR

The general applicability of the *in vivo* panning method to identify tumor homing peptides was examined by performing
15 *in vivo* panning against an implanted mouse melanoma tumor.

Mice bearing a melanoma were produced by implantation of B16B15b mouse melanoma cells, which produce highly vascularized tumors. B16B15b mouse melanoma cells were injected subcutaneously into the mammary fat pad of nude
20 mice (2 months old) and tumors were allowed to grow until the diameter was about 1 cm. *In vivo* panning was performed as disclosed above. Approximately 1×10^{12} transducing units of phage expressing the CX₅C (SEQ ID NO:9), CX₆C (SEQ ID NO:10) or CX₇C (SEQ ID NO:11) library were injected, iv,
25 and allowed to circulate for 4 min. Mice then were snap frozen in liquid nitrogen or perfused through the heart while under anesthesia, tumor tissue and brain (control organ) were removed, and phage were isolated as described above. Three rounds of *in vivo* panning were performed.

The amino acid sequences were determined for the inserts in 89 cloned phage recovered from the B16B15b tumors. The peptides expressed by these phage were represented by two predominant sequences, CLSGSLSC (SEQ ID NO:4; 52% of the clones sequenced) and WGTGLC (SEQ ID NO:18; 25% of the clones; see Table 2). Reinfection of phage expressing one of the selected peptides resulted in approximately three-fold enrichment of phage homing to the tumor relative to brain.

TABLE 2
PEPTIDES FROM PHAGE RECOVERED FROM MOUSE B16B15b MELANOMA

5			
	CLSGSLSC (4*)	GICKDDWCQ (107)	TSCDPSLCE (108)
	KGCGTRQCW (109)	YRCREVLQ (110)	CWGTGLC (111)
	WSCADRTCM (112)	AGCRLKSCA (113)	SRCKTGGLCQ (114)
10	PICEVSRWC (115)	WTCRASWCS (116)	GRCLLMQCR (117)
	TECDMSRCM (118)	ARCRVDPCV (119)	CIEGVLGGC (120)
	CSVANSC (121)	CSSTMRC (122)	SIDSTTF (123)
	GPSRVGG (124)	WWSGLEA (125)	LGTDVVRQ (126)
	LVGVRL (127)	GRPGDIW (128)	TVWNPVG (129)
15	GLLLVVP (130)	FAATSAE (131)	WCCRQFN (132)
	VGFGKAL (133)	DSSLRLP (134)	KLWCAMS (135)
	SLVSFLG (136)	GSFAFLV (137)	IASVRWA (138)
	TWGHRLA (139)	QYREGLV (140)	QSADRSV (141)
	YMFWTSR (142)	LVRRWYL (143)	TARGSSR (144)
20	TTREKNL (145)	PKWLLFS (146)	LRTNVVH (147)
	AVMGLAA (148)	VRNSLRN (149)	

25 * - numbers in parentheses indicate SEQ ID NO:.

Localization of the phage expressing a tumor homing peptide in the mouse organs also was examined by immunohistochemical staining of the tumor and various other tissues (see Example V). In these experiments, 1×10^9 pfu of a control (insertless) phage or a phage expressing the tumor homing peptide, CLSGSLSC (SEQ ID NO:4), were injected, iv, into tumor bearing mice and allowed to circulate for 4 min.

Immunostaining was evident in the melanoma obtained from a mouse injected with phage expressing the CLSGSLSC (SEQ ID NO:4) tumor homing peptide. Staining of the melanoma generally was localized to the blood vessels within the tumor, although some staining also was present in the tumor parenchyma. Essentially no staining was observed in a tumor obtained from a mouse injected with the insertless control phage or in skin or in kidney samples obtained from mice injected with either phage. However, immunostaining was detected in the liver sinusoids and in spleen, indicating that phage can be trapped nonspecifically in organs containing RES.

Using similar methods, *in vivo* panning was performed in mice bearing a SLK human Kaposi's sarcoma. Tumor homing peptides were identified and are disclosed in Table 3. Together, these results demonstrate that the *in vivo* panning method is a generally applicable method for screening a phage library to identify phage expressing tumor homing peptides.

TABLE 3

PEPTIDES FROM PHAGE RECOVERED FROM HUMAN KAPOSI'S SARCOMA

5			
	TDCTPSRCT (150*)	SWCQFEKCL (151)	VPCRFKQCW (152)
	CTAMRNTDC (153)	CRESLKNC (154)	CMEGVKVC (155)
	VTCSRSLMCQ (156)	CNNVGSYC (157)	CGTRVDHC (158)
10	CISLDRSC (159)	CAMVSMED (160)	CYLGVSNV (161)
	CYLVNVDC (162)	CIRSAVSC (163)	LVCLPPSCE (164)
	RHCFSQWCS (165)	FYCPGVGCR (166)	ISCAVDACL (167)
	EACEMAGCL (168)	PRCESQLCP (169)	RSCIKHQCP (170)
	QWCSSRWCT (171)	MFCRMRSCD (172)	GICKDLWCQ (173)
15	NACESAICG (174)	APCGLLACI (175)	NRCRGVSCT (176)
	FPCEGKKCL (177)	ADCRQKPC (178)	FGCVMASCR (179)
	AGCINGLCG (180)	RSCAEPWCY (181)	DTCRALRCN (182)
	KGCGTRQCW (183)	GRCVDGGCT (184)	YRCIARECE (185)
	KRCSSSLCA (186)	ICLLAHCA (187)	QACPMLLCM (188)
20	LDCLSELCS (189)	AGCRVESC (190)	HTCLVALCA (191)
	IYCPGQECE (192)	RLCSLYGCV (193)	RKCEVPGCQ (194)
	EDCTSRFCS (195)	LECVVDSCR (196)	EICVDGLCV (197)
	RWCREKSCW (198)	FRCLERVCT (199)	RPCGDQACE (200)
	CNKTDGDEGVTC (201)		

25

* - numbers in parentheses indicate SEQ ID NO:.

EXAMPLE VII

PREPARATION AND CHARACTERIZATION OF
TUMOR HOMING PEPTIDE/DOXORUBICIN CONJUGATES

This example provides methods for conjugating a moiety
5 such as the chemotherapeutic agent, doxorubicin, to a tumor
homing peptide and for characterizing the conjugation
product.

The peptides CNGRC (SEQ ID NO:8), CDCRGDCFC (SEQ ID
NO:1; RGD-4C; Koivunen et al., *supra*, 1995; Pasqualini et
10 al., *supra*, 1997), CGSLVRC (SEQ ID NO:5) and GACVFSIAHECGA
(SEQ ID NO:19) were synthesized, cyclized under high
dilution and purified to homogeneity by HPLC. Conjugation
of the peptides to doxorubicin (Aldrich; Milwaukee WI) was
performed using 1-ethyl-3-
15 (3-dimethylaminopropyl)carbodiimide hydrochloride (EDC;
Sigma; St. Louis MO) and N-hydroxysuccinimide (NHS;
Sigma) as described (Bauminger and Wilchek, *supra*, 1980;
Harlow and Lane, *supra*, 1988; Hurwitz et al., *supra*, 1975).
Unreacted doxorubicin and peptide were removed from the
20 doxorubicin/peptide conjugates by SEPHADEX G25 column
chromatography using phosphate buffered saline. The
conjugates were lyophilized for storage and were resuspended
in sterile water prior to use.

Doxorubicin concentration of the conjugates (see
25 Example VI) was determined by measuring the optical
absorbance of the solution at 490 nm in a standard
spectrophotometer; this wavelength detects only the
doxorubicin, not the peptides. A calibration curve for
doxorubicin was generated and used to calculate the

concentration prior to use. Conjugation of doxorubicin to the various peptides did not affect this curve. This procedure ensures that each of the administered conjugates contained the same amounts of doxorubicin equivalent.

5 HPLC, mass spectrometry, capillary electrophoresis and NMR analyses were performed to characterize the conjugates. HPLC-fluorescence was performed using an INTERSIL ODS-2 column (4.6 x 150 mm) and a mobile phase composed of 0.08% triethanolamine/0.02% phosphoric acid (85%)/27% acetonitrile
10 at 1 ml/min. Fluorescence detection was performed with excitation at 490 nm and emission at 560 nm wavelength and the retention time (RT) and the area under the curves (AUC) for doxorubicin (dox) and for the major peaks was determined. Each of the conjugates has a unique retention
15 time, depending on the peptide, as follows:
doxorubicin/CDCRGDCFC (SEQ ID NO:1), RT 7.4 min, AUC 26%;
doxorubicin/CNGRC (SEQ ID NO:8), RT 4.7 min, AUC 56%; and
doxorubicin/GACVFSIAHECGA (SEQ ID NO:19), RT 7.7 min, AUC 43%. In comparison, the retention time of doxorubicin is
20 10.6 min and, in the various reactions, the AUC was about 5%.

Capillary electrophoresis (CE; Liu et al., *supra*, 1996) was performed in uncoated fused-silica capillaries with 75 μ m internal diameter and an effective separation length of
25 50 cm. The CE detection system was equipped with an UV absorbance detector and an argon laser emitting at 488 nm. The laser beam is transmitted via a fiber optic cable to the detector and illuminates the capillary window and the fluorescence signal is collected through an emission filter.
30 Conjugation of doxorubicin to the peptides changed the

electrophoretic characteristics of each of the conjugates, indicating that this method can be used as a fast screening method to identify progress of the conjugation reaction.

An alternative method of conjugating doxorubicin to
5 peptides was also used (Nagy et al., *supra* 1996; Nagy et al., *supra* 1998). The doxorubicin/CNGRC conjugate prepared by this method was found to be homogeneous by HPLC. Mass spectrometry showed that the doxorubicin/CNGRC (SEQ ID NO:8) conjugate had one predominant mass number (1192.6), which
10 agreed with the mass predicted from the coupling chemistry (predicted mass 1192.2). Similar results were obtained when the RGD-4C (CDCRGDCFC; SEQ ID NO:1) peptide was coupled to doxorubicin.

One dimensional NMR analysis of the doxorubicin/CNGRC
15 conjugate revealed no evidence of resonances arising from free doxorubicin. Two dimension NMR analysis can allow a determination of the precise molecular structure of the doxorubicin-peptide species.

These results demonstrate that a moiety such as the
20 cancer chemotherapeutic agent, doxorubicin, can be efficiently linked to tumor homing peptides of the invention to produce homogeneous doxorubicin/tumor homing peptide conjugates.

EXAMPLE VIII

TUMOR THERAPY USINGDOXORUBICIN/TUMOR HOMING PEPTIDE CONJUGATES

This example demonstrates that doxorubicin/tumor homing
5 peptide conjugates provide a therapeutic advantage over the
use of doxorubicin, alone, for treating tumors.

Doxorubicin concentration of the conjugates (see
Example VI) was determined by measuring the optical
absorbance of the solution at 490 nm in a standard
10 spectrophotometer; this wavelength detects only the
doxorubicin, not the peptides. A calibration curve for
doxorubicin was generated and used to calculate the
concentration prior to use. Conjugation of doxorubicin to
the various peptides did not affect this curve. This
15 procedure ensures that each of the administered conjugates
contained the same amounts of doxorubicin equivalent.

In addition, the viability of tumor cells obtained from
tumors of mice treated with a tumor homing
peptide/doxorubicin conjugate was compared to that of tumors
20 from mice treated with free doxorubicin. In these
experiments, breast tumor bearing mice were size matched
with regard to the tumors, then treated intravenously with
30 µg equivalent of doxorubicin/RGD-4C (CDCRGDCFC;
SEQ ID NO:1) or of free doxorubicin. Five days after
25 treatment, the mice were euthanized and the tumors were
removed. The tumor pairs were weighed and ground and the
cell suspensions were plated (2 g tumor tissue per 150 mm
plate).

Cell numbers were determined at 24 hours and 7 days after plating. Viability of tumor cells from the tumors of mice receiving the doxorubicin/RGD-4C (CDCRGDCFC; SEQ ID NO:1) conjugate was about 3 fold less than cells from tumors of mice treated with the free doxorubicin. These results demonstrate that administration to a tumor bearing mouse of a conjugate comprising a chemotherapeutic agent linked to a tumor homing molecule is more efficacious than administration of the agent, alone, in reducing the viability of tumor cells.

A. In vitro characterization of cytotoxicity:

MDA-MB-435 human breast carcinoma cells were plated at 1×10^5 cells/well in 96 well plates. Cells were incubated with increasing amounts of doxorubicin, the doxorubicin/RGD-4C (CDCRGDCFC; SEQ ID NO:1) conjugate, or the doxorubicin/GACVFSIAHECGA (SEQ ID NO:19; control) conjugate (0.1 to 10 μ g/well of doxorubicin-equivalent) for either 30 min or overnight. Following incubation, the agents were removed by extensive washing with PBS, then fresh medium added and incubation was continued. The number of surviving cells was determined at 24 hours with crystal violet staining (see Koivunen et al., supra, 1994).

In cells exposed to free doxorubicin, the doxorubicin/RGD-4C (CDCRGDCFC; SEQ ID NO:1) conjugate, or the doxorubicin/GACVFSIAHECGA (SEQ ID NO:19) for 30 min, cell death was present only in the cultures treated with the doxorubicin/RGD-4C (CDCRGDCFC; SEQ ID NO:1) conjugate. However, if the agents were not removed after 30 min, the cells were killed by all of the treatments after 24 hr.

These results indicate that enhanced cellular uptake occurs for the doxorubicin/RGD-4C (CDCRGDCFC; SEQ ID NO:1) conjugate.

B. In vivo characterization of doxorubicin/tumor homing peptide conjugates:

Female 2-month old Balb c nu/nu mice (Harlan Sprague Dawley; San Diego CA) were used for these studies and were cared for according to the Burnham Institute animal facility guidelines. MDA-MB-435 breast carcinoma cells (Price et al., *supra* (1993)) were injected in the mammary fat pad of the nude mice and tumor growth was monitored (Pasqualini et al., *supra*, 1997). Tumors were allowed to grow to a size of about 1 cm³ (about 5% of the mouse's body weight) before starting the treatment experiments, except for the toxicity experiments as discussed below.

Weekly doxorubicin/peptide conjugate or control treatments (5 µg/mouse/week of doxorubicin-equivalent) were administered intravenously. In some experiments, as indicated, a dose of 30 µg/mouse was administered every 3 weeks. Treatment with doxorubicin, alone, is referred to as "dox control" and treatment with doxorubicin conjugated to the non-tumor homing control peptide, GACVFSIAHECGA (SEQ ID NO:19), is referred to as "conjugate control." The results obtained in the doxorubicin control groups as compared to the conjugate control groups were not significantly different. As an additional control, in some experiments the tumor homing peptide was mixed with doxorubicin, without linking, and the mixture was administered to tumor bearing mice. Such treatment produced

results that were not statistically different from those obtained with the above described doxorubicin controls.

Mice were anesthetized with a tribromoethanol-based anesthetic mixture (AVERTIN; Papaioannou and Fox, Lab. Anim. 5 43:189-192 (1993)) before each treatment. Anesthetization facilitated the tail vein injections (final volume, 200 μ l) and allowed precise serial three dimensional tumor size measurements. Tumor volume calculations were based on the equation for the volume of an ovaloid: $V=4/3(\pi abc)$, where 10 a, b, and c are 1/2 of the measured diameters in each of the three dimensions.

At necropsy, MDA-MB-435 tumor-bearing mice treated with the doxorubicin/RGD-4C (CDCRGDCFC; SEQ ID NO:1) conjugate had significantly smaller tumors (t test, $p=0.02$), less 15 spread to regional lymph nodes (t test, $p<0.0001$), a lower incidence of pulmonary metastasis and fewer metastatic lesions (t test, $p<0.0001$) than the doxorubicin control treated mice. All of the mice treated with the doxorubicin/RGD-4C (CDCRGDCFC; SEQ ID NO:1) conjugate 20 survived beyond the time when the doxorubicin control and conjugate control mice had died (Log-Rank test, $p<0.0001$; Wilcoxon test, $p=0.0007$). Essentially the same results were obtained in five separate experiments. These results indicate that a doxorubicin/tumor homing peptide provides a 25 therapeutic advantage over doxorubicin, alone, in reducing the growth of a primary tumor and preventing metastasis of the tumor.

Gross and histopathologic examination was performed on the mice. Many of the tumors in the mice treated with 5 μ g

doxorubicin equivalent of doxorubicin/RGD-4C (CDCRGDCFC; SEQ ID NO:1) presented marked skin ulceration and tumor necrosis, whereas no such signs were observed in doxorubicin control group or conjugate control group. Histopathological analysis disclosed a pronounced destruction of the vasculature in the tumors treated with doxorubicin/RGD-4C (CDCRGDCFC; SEQ ID NO:1) conjugate as compared to the doxorubicin control group.

In a dose escalation experiment, tumor bearing mice were treated with the doxorubicin/RGD-4C (CDCRGDCFC; SEQ ID NO:1) at 30 µg/mouse every three weeks for three cycles and were observed, without further treatment, for an extended period of time. The doxorubicin/RGD-4C (CDCRGDCFC; SEQ ID NO:1) treated mice all remained alive more than 6 months after the doxorubicin control and conjugate control mice had died. The results indicate that treatment with a doxorubicin/tumor homing peptide conjugate can have a curative effect.

Acute toxicity studies also were performed. In these experiments, mice bearing extremely large tumors (about 25% of body weight) were treated with 200 µg/mouse doxorubicin or doxorubicin/RGD-4C (CDCRGDCFC; SEQ ID NO:1) conjugate. All of the mice treated with the doxorubicin/RGD-4C (CDCRGDCFC; SEQ ID NO:1) conjugate survived for longer than one week, whereas all of the doxorubicin control mice had died within 48 hr of treatment. These results suggest that accumulation of the doxorubicin/RGD-4C (CDCRGDCFC; SEQ ID NO:1) conjugate in the large tumors reduced the circulating level of the conjugated doxorubicin, thus reducing its toxicity.

Similar results were obtained using the doxorubicin/CNGRC (SEQ ID NO:8) conjugate. In each of three series of experiments, tumors in the mice treated with doxorubicin/CNGRC (SEQ ID NO:8) were significantly smaller than tumor is the doxorubicin control and conjugate control groups. Treatment with the doxorubicin/CNGRC (SEQ ID NO:8) conjugate almost completely suppressed tumor growth, whereas free doxorubicin and doxorubicin conjugated to the control peptide had essentially no effect on tumor growth relative to treatment with the vehicle, alone. A marked effect on survival also was observed and some of the doxorubicin/CNGRC (SEQ ID NO:8) treated animals survived for extended periods of time (Log-Rank test, $p=0.0064$; Wilcoxon test, $p=0.0343$). In addition, the doxorubicin/CNGRC (SEQ ID NO:8) conjugate was less toxic than free doxorubicin. These results confirm that conjugates comprising a chemotherapeutic agent and a tumor homing molecule provide a therapeutic advantage in treating cancer.

EXAMPLE IX

20 INTERACTION OF CD13/APN WITH NGR LIGANDS

This example describes the interaction of CD13/APN with NGR containing peptides.

The use of phage display peptide libraries to isolate minimal receptor sequences that bind to fibronectin and RGD-containing fibronectin fragments in affinity panning is described in Pasqualini et al., J. Cell Biol. 130:1189-1196 (1995), which is incorporated herein by reference. A predominant motif, CWDD(G/L)WLC was obtained and shown to be a structural mimic of an RGD-binding site on integrins

(Pasqualini et al., *supra*, 1995). By searching the protein database for sequences homologous to the W(D/N)DGWL sequence (an RGD-binding peptide, Pasqualini et al., *supra*, 1995), an identical peptide sequence, except for the flanking
5 cysteines, was found in aminopeptidases. The W(D/N)DGWL sequence was highly conserved in aminopeptidases from bacteria to humans, indicating that this motif represents a functionally relevant domain (Favaloro et al., *supra* (1988); Rawlings and Barret, Biochem J. 290:205-218 (1993), each of
10 which is incorporated herein by reference).
CD13/Aminopeptidase N contains the closely related WNDGWL sequence (Look et al., *supra* (1989); Chen et al., J. Immunol. 157:2593-2600 (1996), each of which is incorporated herein by reference).

15 Binding of CNGRC-phage to Immobilized CD13/APN

Given that the NGR sequence closely resembles a reverse RGD sequence, NGR-containing phage were tested to determine whether they would bind to CD13/aminopeptidase (CD13/APN). Kaposi's sarcoma tumor cell octylglucoside extracts were
20 used as a source of CD13/APN. Aminopeptidase enzymatic activity was measured in these extracts utilizing Ala-pNA or Leu-pNA as substrates by monitoring the absorbance of the samples at 405 nm (Look et al., *supra* (1989); Taylor, *supra* (1993); Amoscato et al., *supra* (1989), each of which is
25 incorporated herein by reference). The specificity was confirmed by demonstrating inhibition by o-phenanthroline, a specific inhibitor of aminopeptidase N.

Figure 4A shows that aminopeptidase activity from the Kaposi's sarcoma cell extracts binds specifically to

microtiter wells coated with the CD13 antibody, but not to wells coated with BSA. The CD13 antibody alone had no aminopeptidase activity. Moreover, the aminopeptidase N inhibitor, o-phenanthroline, inhibited the activity from the 5 tumor extracts captured by the antibody.

In Figure 4B, NGR-containing phage were assayed for the ability to bind CD13. CD13 from Kaposi's sarcoma tumor cell octylglucoside extract was bound to microtiter wells precoated with the anti-CD13 antibody WM15 (Pharmingen; San 10 Diego CA) (Favaloro et al., Br. J. Haematol. 69:163-171 (1988), which is incorporated herein by reference). Various phage were added to the wells at 2×10^9 transducing units (TU) per well. The bound phage were quantitated by infection of bacteria as described (Koivunen et al., *supra* 15 (1993); Pasqualini et al., *supra*, 1995). The data were expressed as means and standard errors from triplicate wells. As shown in Figure 4B, three different phage, each of which displays the NGR motif in a different context, showed strong and selective binding to the immobilized CD13. 20 Two RGD phage showed no significant binding (Figure 4B), and various unrelated control phage also did not bind. These results indicate that CD13 recognizes the NGR motif better than RGD.

To confirm the specificity of NGR binding by CD13, the 25 ability of NGR soluble peptides to inhibit the interaction was studied. Either CNGRC (SEQ ID NO:8), CRGDGWC (SEQ ID NO:220), or GACVFSIAHECGA (SEQ ID NO:19) (unrelated control) peptides were added at 200 μ g/well to the phage binding assay, and the bound phage quantitated by infection of

bacteria. The data shown were expressed as means and standard errors from triplicate wells.

As shown in Figure 4C, the NGR cyclic peptide, but not the RGD or the unrelated peptide, blocked the NGR phage binding. These results demonstrate that phage expressing the peptide sequence NGR and NGR peptides bind specifically to a CD13 related molecule.

Phage Binding Assays with CD13/APN-transfected Cells

The CNGRC phage binds specifically to cells transfected with CD13. Two cell lines that do not express endogenous CD13, the Molt-4 human T cell leukemia and MDA-MB-435 breast carcinoma cell lines, were used to assay CNGRC (SEQ ID NO:8) phage binding to cells transfected with CD13. Briefly, mock transfected Molt-4 cells ("Molt-4/mock") and cells transfected with CD13 ("Molt-4/CD13") were incubated with CNGRC (SEQ ID NO:8) phage or with control Fd phage lacking an insert (2×10^9 transducing units (TU) per sample). Synthetic peptides, CNGRC (SEQ ID NO:8) or the negative control CARAC (SEQ ID NO:221), were pre-incubated with the cells at 250 and 500 $\mu\text{g/ml}$. After extensive washing, bound phage were quantitated by infection of bacteria as described above. As shown in Figure 5, CNGRC phage were found to bind to Molt-4/CD13 and to MDA-MB-435/CD13 transfected cells, but not to parental cell lines transfected with an empty control vector. Furthermore, the binding was blocked by the CNGRC (SEQ ID NO:8) peptide, but not by control peptides, demonstrating that the CNGRC (SEQ ID NO:8) binding was specific (Figure 5). These results indicate that CD13 gene transfer confers to cells the ability to bind NGR phage.

EXAMPLE X

AFFINITY PURIFICATION OF AN NGR RECEPTOR AND SCREENING FOR
HIGH AFFINITY LIGANDS

This example describes affinity purification of an NGR
5 receptor and screening for high affinity ligands of an NGR
receptor.

Affinity Chromatography of Extracts from CD13/APN Positive
Cells on Immobilized CNGRC Peptide Yields CD13/APN

To further characterize the interaction of CD13/APN
10 with the NGR peptide, affinity chromatography on the CNGRC,
peptide was performed. The column was prepared by coupling
the CNGRC peptide to SEPHAROSE (Pharmacia; Piscataway NJ).
Elution was effected with soluble CNGRC peptide.
Experiments were performed using HL-60 cell extracts because
15 they are known to express CD13/APN without other proteases
that could interfere in the APN assay (Xu et al., *supra*
(1997)). Thus, in HL-60 cell extracts, all detectable cell
surface enzymatic activity is inhibitable by anti-CD13
blocking antibodies, assuring specificity. Extracts of
20 Molt-4 and MDA-MB-435 cells transfected with CD13/APN were
also characterized by using affinity chromatography, with
extracts of the mock transfected counterparts used as
negative controls.

Briefly, CD13 was detected by testing aliquots of each
25 fraction for the presence of CD13 immunoreactivity in ELISA
with the anti-CD13 monoclonal antibody WM15, using normal
mouse IgG as a negative control. The fractions were also
analyzed for CD13 enzymatic activity using AlapNA as the

substrate. In all cases, using Molt-4/CD13, MDA-MB-435/CD13 or HL-60 cell extracts, elution of the CNGRC-column with the CNGRC peptide yielded fractions containing functional CD13/APN (Figure 6). No CD13 immunoreactivity was observed in the control samples using IgG, and no enzymatic activity was found employing a MMP-specific substrate. Moreover, affinity chromatography on a control peptide (CARAC; SEQ ID NO:221) yielded no CD13/APN, and the CARAC peptide did not elute any activity from the CNGRC column. Furthermore, no CD13 activity was recovered from the CNGRC column when the extract fractionated was derived from CD13-negative cells (see Figure 6).

Isolation of Endothelial NGR receptors by Affinity Chromatography

Protocols based on those developed for the isolation of certain integrins are used to isolate an NGR receptor using affinity chromatography. Tumor extracts and cultured endothelial cells treated with TNF α , a known angiogenic stimulator, prepared by extraction with octylglucoside are used as the source of the receptor. The presence of the receptor is confirmed using a phage binding assay and immunoblot analysis as described above. The specific phage show more binding to the activated endothelial cells than control phage. Cell binding assays with phage are performed as described previously (Barry et al., *supra* (1996), which is incorporated herein by reference).

The extract is run over a column of the peptide, coupled to SEPHAROSE, and bound material is eluted with the soluble peptide at 1 mg/ml. High affinity peptides isolated

during the screening of secondary NGR libraries, described above, are used. Cells that do not bind the NGR-containing phage are used to make a control extract, and control elution from the peptide column is carried out with an unrelated cyclic peptide. The eluates are probed with anti-CD13 and the anti-NGR receptor phage antibodies. If desired, anti-NGR receptor antibodies are used for affinity chromatography alternatively or consecutively with the peptide affinity chromatography. The NGR receptor preparations obtained by NGR peptide affinity isolation contain an NGR reactive fragment and are reactive with CD13 antibodies.

Screening of secondary NGR libraries to identify high affinity ligands for the NGR receptor

In order to identify additional high affinity ligands for the NGR receptor, secondary NGR libraries are constructed. Three such libraries, $X_2\text{CNGRCX}_2$ (SEQ ID NO:222), $CX_2(C/X)\text{NGR}(C/X)X_2C$ (SEQ ID NO:223), and CNGRCX_6 (SEQ ID NO:224) (where "C" is cysteine and "X" is any amino acid), were prepared. In these secondary libraries, the NGR sequence is represented in different contexts which allows for multiple folding arrangements. Because higher affinities can be achieved with cyclic peptides, the libraries feature the NGR tripeptide in different cyclic configurations. The secondary libraries are screened against the target receptor in order to select the best-fit sequence/conformation for the ligand. This has been empirically demonstrated in a number of systems (Sparks et al., Methods Enzymol. 255:498-509 (1995); Martens et al., J. Biol. Chem. 270:21129-21136 (1995); Wrighton et al., Science

273:458-464 (1996), each of which is incorporated herein by reference).

The secondary NGR phage peptide libraries are constructed as follows. Phage display libraries are made as described in Smith and Scott, Science 228:1315-1317 (1985); Smith and Scott, Methods Enzymol. 21:228-257 (1993); and Koivunen et al., Methods Enzymol. 245:346-349 (1994), each of which is incorporated by reference). Briefly, FUSE 5 plasmid is isolated from transfected MC1061 cells using Qiagen Maxi-prep 500 columns (Qiagen; Chatsworth CA). The plasmid is cleaved to remove the 14 bp "stuffer" using SfiI restriction enzyme (New England Biolabs; Beverly MA) and purified with QIAquick PCR purification columns (Qiagen). The oligonucleotides encoding the peptides which are to be displayed by the phage are purchased commercially and converted into double stranded DNA utilizing a library primer (5'-TTCTGCCCCAGCGGCCCC-3'; SEQ ID NO:202). Two μ g of the oligonucleotide and 4 μ g of the primer are annealed in a volume of 10 μ l at 65°C for 2 min and cooled to room temperature. Primer extension is performed with SEQUENASE 2.0 DNA polymerase (United States Biochemical) for 60 min at 37°C in a reaction volume of 50 μ l containing 10 mM, and 5 mM dithiothreitol, and the double stranded oligonucleotides are purified using the QIAquick nucleotide removal kit (Qiagen). The oligonucleotide is subsequently digested with BglI (Boehringer Mannheim; Indianapolis IN) overnight at 37°C, purified as described above, and ligated to the FUSE 5 vector with T4 DNA Ligase (Gibco-BRL; Gaithersburg MD). The plasmid is then transfected into MC1061 bacteria utilizing the Cell-Porator apparatus (Gibco-BRL). Approximately 25 μ l of bacteria and ~100 ng of plasmid are shocked with 2.5 kV

- and immediately transferred to S.O.C. medium. A total of 100-200 electroporations are performed in order to achieve $\sim 10^9$ clones. For preparation of a CX₂CNGRCX₂ (SEQ ID NO:222) secondary library, the primer 5'-CACTCGGCCGACGGGGCTTGTNNK
- 5 NNKTGTAATGGTAGGTGTNNKNNKGGGGCCGCTGGGGCAGAA-3' (SEQ ID NO:202) is used, where N is any nucleotide and K is T or C). For preparation of a CX₂(C/X)NGR(C/X)X₂C (SEQ ID NO:223) secondary library, the primer 5'-CACTCGGCCGACGGGGCTTGTNNKNNKNNK
- 10 AATGGGAGGNNKNNKNNKGGGGCCGCTGGGGCAGAA-3' (SEQ ID NO:203) is used. For preparation of a CNGRCX₆ (SEQ ID NO: 224) secondary library, primer 5'-CACTCGGCCGACGGGGCTTGTAAATGGGAGATGTNNKNNKNNKNNKNNKNNKGGGGCCGCTGGGGCAGAA-3' (SEQ ID NO:204) is used. The secondary NGR phage libraries are screened for
- 15 the ability to bind immobilized CD13 or against CD13 transfected cells as described above, and binding ligands are identified. The secondary NGR libraries can also be screened for homing to tumor or other angiogenic vasculature.
- 20 Screening of combinatorial scFv libraries from rabbits immunized with human tumor xenografts

In order to identify additional high affinity ligands for the NGR receptor, combinatorial scFv libraries are prepared from rabbits immunized with human tumor xenografts.

25 The tumor xenografts are established as described in Pasqualini et al., *supra*, 1997, and Arap, *supra*, 1998. The tumors are homogenized in PBS for immunization and injected subcutaneously into rabbits every two weeks. At least four injections are given; the immune response of the rabbits is

30 evaluated by immunostaining of tumor tissue sections after

the second and third immunization. Antibodies from sera of immunized rabbits are tested for binding to immobilized CD13, and to human integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ as positive controls. Spleen and bone marrow from the immunized rabbits are harvested, and tissues from identically treated rabbits are pooled and used for total RNA preparation with TRI reagent (Molecular Research Center; Cincinnati OH). Reverse transcription of total RNA is performed with the 1st Strand cDNA Synthesis Kit for RT PCR (AMV; Boehringer Mannheim).

10 Using the PCR primers listed below, V_k (nine primer combinations), V_l (one primer combination), and V_g (four primer combinations) encoding sequences are amplified from the first strand cDNA. In pilot studies, all combinations gave rise to PCR products in the range of 350 bp. Using the

15 overlap extension PCR primers listed below, scFv encoding sequences are assembled by fusing V_k and V_l , respectively, with V_g following SfiI cloning of the scFv encoding sequences into the phagemid vector pComb3H. Selection of the resulting scFv libraries against immobilized CD13 is

20 performed.

The following primers are used for V_k amplification (F = forward primer, B= backward primer; sequences in 5' to 3' direction; M = A/C, R = A/G, W = A/T, S = C/G, Y = C/T, K = G/T, V = A/C/G, H = A/C/T, D = A/G/T, B = C/G/T, N = A/C/G/T) :

25 RSCVK1-F (GGGCCCAGGCGGCCGAGCTCGTGMTGACCCAGACTCCA;
SEQ ID NO:205);
RSCVK2-F (GGGCCCAGGCGGCCGAGCTCGATMTGACCCAGACTCCA;
SEQ ID NO:206);

30 RSCVK3-F (GGGCCCAGGCGGCCGAGCTCGTGATGACCCAGACTGAA;
SEQ ID NO:207);

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- RKB9J0-B (GGAAGATCTAGAGGAACCACCTTTGATTTCCACATTGGTGCC; SEQ ID NO:208);
- RKB9J10-B (GGAAGATCTAGAGGAACCACCTTTGATTTCCACATTGGTGCC; SEQ ID NO:209); and
- 5 RKB42J0-B (GGAAGATCTAGAGGAACCACCTTGACSAACCTCGGTCCC; SEQ ID NO:210).

The following primers are used for V₁ amplification:

- RSCλ1 (GGGCCCAGGCGGCCGAGCTC GTGCTGACTCAGTCGCCCTC; SEQ ID NO:211) and RJλ0-B
- 10 (GGAAGATCTAGAGGAACCACCGCCTGTGACGGTCAGCTGGGTCCC; SEQ ID NO:212).

The following primers are used for V₂ amplification:

- RSCVH01 (GGTGGTTCCTCTAGATCTTCCCAGTCGGTGGAGGAGTCCRG; SEQ ID NO:213);
- 15 RSCVH02 (GGTGGTTCCTCTAGATCTTCCCAGTCGGTGAAGGAGTCCGAG; SEQ ID NO:214);
- RSCVH03 (GGTGGTTCCTCTAGATCTTCCCAGTCGYTGGAGGAGTCCGGG; SEQ ID NO:215);
- 20 RSCVH04 (GGTGGTTCCTCTAGATCTTCCCAGSAGCAGCTGRTGGAGTCCGG; SEQ ID NO:216); and
- RSCG-B (CCTGGCCGGCCTGGCCACTAGTGACTGAYGGAGCCTTAGGTTGCCC; SEQ ID NO:217).

- The followings primers are used to prepare the scFv
- 25 fusion:

RSC-F (GAGGAGGAGGAGGAGGAGGCGGGGCCAGGCGGCCGAGCTC; SEQ ID NO:218) and

RSC-B (GAGGAGGAGGAGGAGGAGCCTGGCCGGCCTGGCCACTAGTG; SEQ ID NO:219).

EXAMPLE XI

Selective Cytotoxicity of Doxorubicin-CNGRC Conjugate
Administered to CD13/APN-positive Cells

An *in vitro* bioassay was developed to measure receptor
5 binding of CNGRC-doxorubicin (CNGRC-dox) (CNGRC; SEQ ID
NO:8) conjugates to cells. Endothelial cells activated with
TNF α were used. Alternatively, the test cells were
transfected with CD13/APN. This assay is based on the fact
that, after a brief incubation period, drug that has not
10 bound to the cells can be removed by washing. There is a
quantifiable difference in the cytotoxic effect of the
doxorubicin conjugate compared to doxorubicin alone or
doxorubicin coupled to a control peptide after washing,
because the drug remains bound to the cells if the receptor
15 for the conjugated peptide is present.

This system was used with activated endothelial cells
because CD13 expression has been shown to be upregulated
when endothelial cells such as human umbilical vein
endothelial cells (HUVEC) are stimulated (see Figure 7). It
20 was observed that CD13 was induced upon stimulation of
endothelial cells with growth factors such as bFGF, VEGF and
TNF α , known endothelial cell activator cytokines.

Briefly, to study the upregulation of CD13 by
angiogenic factors, endothelial cells were cultured in
25 different conditions, as indicated in Figure 7, and the cell
surface was stained for CD13 with the anti-CD13 monoclonal
antibody, WM15. The percentage of CD13 expression was
calculated based on the levels detected in cells growing in
normal tissue culture medium with 10% FCS.

In vitro cytotoxicity of doxorubicin and targeted doxorubicin was evaluated using activated HUVECs (Figure 8A) or CD-13 transfected 435-breast carcinoma cells (Figure 8B). These cell lines are sensitive to doxorubicin. Cell
5 monolayers were incubated with doxorubicin or a doxorubicin-peptide conjugate of RGD-4C (CDCRGDCFC; SEQ ID NO:1), CNGRC (SEQ ID NO:8), or CARAC (SEQ ID NO:221) at a concentration of 1 μ g/well for 20 minutes in two sets of triplicates. One set of triplicates was washed three times
10 with DMEM to remove unbound drug 20 min after drug addition (right). Untreated monolayers were used as controls. After 24 hours, attached cells were fixed and quantified by Crystal Violet.

The CNGRC-doxorubicin conjugate was more effective on
15 the CD13-expressing cells than the control cells (see Figure 8). These results demonstrate that CNGRC-doxorubicin has CD13-dependent cytotoxic activity *in vitro* and that the CNGRC (SEQ ID NO:8) peptide can guide a toxic moiety such as doxorubicin to CD13-positive cells.

20

EXAMPLE XII

CD13 EXPRESSION IN MOUSE AND HUMAN ANGIOGENIC VASCULATURE

These experiments demonstrate that CD13 is expressed in angiogenic vasculature but is not detected in normal vasculature.

25 As described above, NGR-containing phage injected intravenously home to tumor vasculature, but not to normal vessels in other tissues. To determine binding of CNGRC

(SEQ ID NO:8) phage to tissues using a different technique and to analyze human tissue, immunohistochemical staining of CNGRC (SEQ ID NO:8) phage and Fd control phage in tissue sections was performed. Phage displaying the peptide CNGRC
5 (SEQ ID NO:8) or control phage with no insert were incubated with human breast carcinoma and normal breast tissue. An antibody against M-13 phage (Pharmacia) was used for the staining. The results indicated that the CNGRC-phage binds to human tumor vessels in tissue sections, but not to normal
10 vessels. A phage with no insert (fd phage), used as a negative control, did not bind to normal or to tumor tissue sections.

As shown in Table 4, CD13 was expressed in a number of tumor cells and HUVECs. However, CD13 was not detected in
15 the vasculature of normal organs (Table 5). Thus, based on the analysis of CD13 expression in the vasculature in murine and human tissues, CD13 is expressed in angiogenic vessels, but cannot be detected in normal vessels.

Table 4		
Expression of CD13/APN on cell lines		
<u>anti-CD13 monoclonals</u>	<u>WM15</u>	<u>1H4</u>
MDA-MB-435	-	-
5 (Breast carcinoma)		
KS1767	++++	+++
(Kaposi's sarcoma)		
C8161	++++	++++
(melanoma)		
10 SKOV3-ip	+	+
(ovarian carcinoma)		
HUVECs	-/++/++++	-/+++
(endothelial cells)		
HDM-ZW	+++++	++++
15 (Hodgkin's lymphoma)		
Molt-4	-	-
(T cell leukemia)		
HL-60	+++	+++
(acute myeloid leukemia)		

20 Immunostainings were performed using two monoclonal antibodies against CD13, WM15 and 1H4.

Table 5			
Expression of CD13/APN in normal and tumor vasculature			
	<u>Anti-CD13 monoclonals</u>	<u>WM15</u>	<u>1H4 ab</u>
	blood vessels in normal organs		
5	brain	-	-/+
	kidney	-	-
	skin	-	-
	liver	-	-
	lung	-	-
10	spleen	-	-
	intestine	-	-
	heart	-	-
	retina	-	-
	spinal cord	+/-	+/-
15	tumor vasculature*		
	endothelial cells	++++	++++
	pericytes	++++	+++

*Human tumors tested: breast, colon, gastric, and esophageal carcinomas. Analysis of normal mouse tissues and tumors
 20 (MDA-MB-435 and Kaposi's sarcoma) with anti-mouse anti-CD13 antibodies (R3-63 and 2M7) showed similar results.

Confocal microscopy was performed to study the cellular localization of CD13 in tumor vasculature. Confocal
 microscopy was used to detect immunofluorescent staining of
 25 CD13 in tumor vessels. The images were obtained by superimposing the immunofluorescent and differential interference contrast (DIC) images, to reveal tissue

morphology, or by direct immunofluorescence. Human breast carcinoma tissue sections were incubated with an anti-CD13 antibody (WM15) or control IgG. A FITC-conjugated secondary antibody against mouse IgG was used to reveal anti-CD13 localization. The negative control omitted the primary antibodies. Human breast carcinoma serial sections, gastric adenocarcinoma and colon carcinoma tissue sections were stained with anti-CD13 antibody. An antibody against APA, a known pericyte marker, was used for localization purposes.

10 Autofluorescence of fixed red blood cells (RBC) was visible. In tissue sections incubated with the anti-CD13 monoclonal antibody, only endothelial cells on small tumor vessels showed specific immunoreactivity. CD13 expression was confined to the endothelial cells and pericytes in

15 multiple tumor types including breast, colorectal, and gastric carcinoma. Similar staining patterns were obtained with the NGR phage. In each case, reactivity could be detected in the vasculature of the tumors, but not in the vasculature of normal tissues (see Table 5). As expected,

20 APA was not detected in endothelial cells but was present in pericytes located in tumor blood vessels. These results demonstrate that CD13 is expressed in angiogenic vasculature but is not detected in normal vasculature.

EXAMPLE XIII

25 NGR PHAGE HOME PREFERENTIALLY TO TUMOR VASCULATURE OVER
RETINAL NEOVASCULATURE

These results demonstrate that NGR phage preferentially home to tumor vasculature compared to retinal vasculature.

One experimental model of angiogenesis, oxygen- induced angiogenesis, was used to evaluate the role of CD13 in angiogenesis (Pierce et al., Proc. Natl. Acad. Sci. USA 92:905-909 (1995); Smith et al., Invest. Ophthalmol. Vis. Sci. 35:101-111 (1994), each of which is incorporated herein by reference). NGR phage bound to angiogenic retinal neovasculature induced by oxygen, giving results similar to those obtained with RGD-4C (CDCRGDCFC; SEQ ID NO:1) phage. CNGRC, RGD-4C (CDCRGDCFC; SEQ ID NO:1), and control fd phage lacking an insert were recovered from breast carcinoma xenografts, normal retina or angiogenic retina. Briefly, 10^6 transducing units (TU) of phage were injected into the tail vein of mice bearing size-matched MDA-MB-435 derived tumors ($\sim 1000 \text{ mm}^3$) or p17 mice that had been exposed to oxygen (Pierce et al., *supra*, 1995; Smith et al., *supra*, 1994). The same number of transducing units of an ampicillin control phage was co-injected in order to assess specificity of the different tetracycline phage. In each case, phage were recovered after perfusion. The ratios were calculated using the mean number of phage recovered from the tissues after triplicate plating on ampicillin selective and tetracycline selective plates.

Mice that were not exposed to oxygen did not develop retinal angiogenic vessels (control) and were also injected to evaluate phage homing to normal retina. One-week-old C57BL/6J mice were exposed to a 75% oxygen atmosphere for five days, and then kept in room air for another five days (Figure 9A). The proliferative neovascular response was quantified by counting the nuclei of new vessels extending from the retina into the vitreous in $6 \mu\text{m}$ cross-sections. This model was used to assess the binding of phage injected

intravenously to the newly formed angiogenic vessels. To test phage homing in this system, phage displaying the RGD-4C (CDCRGDCFC; SEQ ID NO:1) peptide (which binds selectively to α_v integrins) or CNGRC-phage were injected intravenously prior to immunohistochemical analysis. The α_v integrins have been shown to be upregulated in these vessels. Phage staining was seen with the RGD-4C (CDCRGDCFC; SEQ ID NO:1) and CNGRC phage in the neovascular formations, whereas the normal vessels were negative.

10 Side by side comparison of the RGD-4C (CDCRGDCFC; SEQ ID NO:1) and the CNGRC-phage homing in angiogenic vasculature of retina or tumors indicated that the two phage have opposite homing preferences: more RGD-4C (CDCRGDCFC; SEQ ID NO:1) phage were recovered from the retina than
15 CNGRC-phage, whereas more CNGRC-phage were recovered from tumors (Figure 9B). The homing of each phage also was inhibited by the appropriate cognate peptide, and this inhibition was not observed with control phage (Figure 9B). These results indicate that expression of the NGR receptor
20 can be higher during tumor angiogenesis than in other types of angiogenesis.

EXAMPLE XIV

ROLE OF CD13 IN ANGIOGENESIS

These experiments demonstrate that CD13 is functionally
25 important in angiogenesis.

Several experimental models involving cytokine-induced angiogenesis (Brooks et al., Cell 79:1157-1164 (1994) and tumor-induced angiogenesis (Folkman, *supra* (1995); Arap et

al., *supra* (1998), each of which is incorporated herein by reference) were used to evaluate the role of CD13 in angiogenesis.

A functional role for CD13 in angiogenesis was demonstrated by using CD13 antagonists. Specifically, the CD13 inhibitor bestatin, as well as monoclonal antibodies that block CD13 enzymatic activity, were tested in several angiogenesis assays. These assays included cytokine-induced angiogenesis in the CAM, oxygen-induced retinal neovascularization, and tumor-induced angiogenesis.

Oxygen-induced angiogenesis in the retina was inhibited by treating the animals with antagonists of CD13. Briefly, inhibition of oxygen-induced retinal neovascularization was determined upon injection of CD13 antagonists. One-week-old C57BL/6J mice were exposed to a 75% oxygen atmosphere for five days, and then kept in room air for one week. In the control mice, which were treated with PBS or rat IgG, a proliferative neovascular response resulted and was quantified as described above. Anti-mouse CD13 inhibitory antibodies R3-63 and 2M7 (250 μ g/mouse/day) or bestatin (200 μ g/mouse/day) were injected intraperitoneally, and the inhibitory effect determined. Animals treated with the CD13 antagonist antibodies and bestatin showed only 20-30% of the angiogenic response observed in the mice treated with controls (IgG and vehicle).

The CD13 antagonists also suppressed bFGF-induced angiogenesis in the CAM. Briefly, angiogenesis was induced in CAMs from 10-day chicken embryos by bFGF filters implanted onto regions that were previously avascular.

Various treatments were applied topically, and, after 3 days, the filters and surrounding CAMs were resected and fixed in formalin.

Figure 10A shows the number of blood vessels entering the disk within the focal plane of the CAM, counted under a stereomicroscope by two observers in a double-blind fashion. Each bar represents the mean number of vessels and standard errors from 8 CAMs on each group. There were significantly fewer vessels entering the disks treated with inhibitors of CD13 enzymatic activity ($p < 0.05$). These results also demonstrate that the inhibitory antibody used in the anti-angiogenic treatments recognizes chicken CD13 and that CD13 is expressed in CAM angiogenic vasculature.

The effects of anti-mouse CD13 agonists were also studied in tumor-bearing mice. Mice bearing size-matched 435 breast carcinoma tumors were divided in three groups (five mice per group). Anti-mouse CD13 inhibitory antibodies R3-63 and 2M7 (500 $\mu\text{g}/\text{mouse}/\text{day}$) or bestatin (200 $\mu\text{g}/\text{mouse}/\text{day}$) were injected intraperitoneally. In the control groups, mice were treated with PBS or rat IgG. As shown in Figure 10B, tumor growth was retarded in comparison with the tumor growth seen in mice that received control rat IgG or injections of vehicle only. Treatment with bestatin produced similar results to those observed with the anti-CD13 antibodies ($p < 0.05$).

These results indicate that CD13 is a new marker of tumor vasculature that serves as a specific receptor for NGR ligands and plays a functional role in angiogenesis.

EXAMPLE XV

TUMOR THERAPY USING DOXORUBICIN/TUMOR HOMING NGR PEPTIDE
CONJUGATES

This example describes *in vivo* tumor therapy with NGR
5 peptide conjugated to doxorubicin.

The drug conjugates made with RGD-4C (CDCRGDCFC; SEQ ID
NO:1) and CNGRC were found to be more effective and less
toxic than the free drug (Arap et al., *supra* (1998), which
is incorporated herein by reference). Similar results were
10 observed when mice bearing multiple tumor types were treated
with the drug-conjugates. Specifically, mice bearing MDA-
MB-435-derived breast carcinomas and Hodgkin's lymphoma were
treated with doxorubicin-CNGRC peptide conjugate as follows.

Briefly, mice with size-matched tumors (~1000 mm³) were
15 randomized into four treatment groups (six animals per
group): vehicle only, free doxorubicin, doxorubicin-control
peptide, and doxorubicin-CNGRC (SSEQ ID NO:8). Mice were
treated at 5 µg/mouse/week of doxorubicin-equivalent, and
the difference in tumor volumes between day 1 and day 28
20 determined (Figure 11A). Figure 11B shows a Kaplan-Meier
survival curve of the mice in Figure 11A.

To test toxicity of the CNGRC conjugate, mice bearing
large (~5000 mm³) MDA-MB-435 breast carcinomas (four animals
per group) were randomized to receive a single-dose of free
25 doxorubicin or doxorubicin-CNGRC conjugate at 200 µg/mouse
of doxorubicin equivalent. The data are shown as a Kaplan-
Meier survival curve in Figure 11C.

Mice bearing large (~5000 mm³) Hodgkin's lymphoma (eight animals per group) were randomized to receive two doses of free doxorubicin plus unconjugated CNGRC peptide or doxorubicin-CNGRC (SEQ ID NO:8) conjugate at 40 µg/mouse of
5 doxorubicin equivalent. The data are shown as a Kaplan-Meier survival curve in Figure 11D.

The experiments show the effect of doxorubicin-CNGRC conjugate on mice bearing breast carcinomas (Figure 11A, 11B and 11C) (Arap et al., *supra*, 1998), and Hodgkin's lymphomas
10 (Figure 11D). Tumor-bearing mice treated with doxorubicin-CNGRC (SEQ ID NO:8) lived longer than those treated with the unconjugated peptide doxorubicin mixture or doxorubicin alone (Figure 11B, 11C and 11D) (Arap et al., *supra*, 1998). Cures were also observed, which was not seen with the
15 control treatments. Similar results were obtained when the doxorubicin-CNGRC (SEQ ID NO:8) conjugate was tested in melanoma and Kaposi's sarcoma xenografts. Furthermore, the targeting of the CNGRC-phage and the anti-tumor effect of the doxorubicin-CNGRC (SEQ ID NO:8) conjugate *in vivo* was
20 equally efficient, whether or not the tumor cells expressed CD13/NGR receptor as in the case of Hodgkin's lymphoma, C8161 melanoma, and Kaposi's sarcoma or did not express CD13 as in MDA-MD-435 breast carcinoma.

These results demonstrate that it is possible to target
25 a chemotherapeutic drug to angiogenic vasculature, a common feature in all solid tumors, using an NGR tumor homing peptide that homes to an NGR receptor.

Although the invention has been described with reference to the disclosed examples, it should be understood

that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

We claim:

1. A method of identifying a tumor homing molecule that homes to angiogenic vasculature of a tumor, comprising:

(a) contacting a substantially purified NGR receptor
5 with one or more molecules; and

(b) determining specific binding of a molecule to said NGR receptor,

wherein the presence of specific binding identifies said molecule as a tumor homing molecule that homes to angiogenic
10 vasculature of a tumor.

2. The method of claim 1, further comprising the steps of:

(c) administering an NGR binding molecule *in vivo*; and

(d) determining binding of said NGR binding molecule to
15 angiogenic vasculature of a tumor.

3. The method of claim 1, wherein said substantially purified NGR receptor is CD13/aminopeptidase N.

4. The method of claim 1, wherein said substantially purified NGR receptor is immobilized to a support.

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5. A method of identifying a homing molecule that homes to angiogenic vasculature, comprising:

(a) contacting a substantially purified NGR receptor with one or more molecules; and

5 (b) determining specific binding of a molecule to said NGR receptor,

wherein the presence of specific binding identifies said molecule as a homing molecule that homes to angiogenic vasculature.

10 6. The method of claim 5, further comprising the steps of:

(c) administering an NGR binding molecule *in vivo*; and

(d) determining binding of said NGR binding molecule to angiogenic vasculature.

15 7. The method of claim 5, wherein said substantially purified NGR receptor is CD13/aminopeptidase N.

8. The method of claim 5, wherein said substantially purified NGR receptor is immobilized to a support.

9. A method of directing a moiety to angiogenic vasculature of a tumor in a subject, comprising administering to the subject a conjugate comprising a moiety linked to a tumor homing molecule that exhibits specific
5 binding to an NGR receptor, whereby the moiety is directed to angiogenic vasculature of a tumor.

10. The method of claim 9, wherein said tumor homing molecule is a peptide containing the sequence NGR.

11. The method of claim 10, wherein said moiety is a
10 cytotoxic agent.

12. The method of claim 10, wherein said moiety is a drug.

13. The method of claim 12, wherein said drug is a cancer chemotherapeutic agent.

14. The method of claim 13, wherein said cancer
15 chemotherapeutic agent is doxorubicin.

15. The method of claim 10, wherein said tumor homing peptide comprises a sequence selected from the group consisting of CNGRCVSGCAGRC (SEQ ID NO:3), NGRAHA (SEQ ID
20 NO:6), CVLNGRMEC (SEQ ID NO:7), and CNGRC (SEQ ID NO:8).

16. The method of claim 15, wherein said moiety is a cytotoxic agent.

17. The method of claim 15, wherein said moiety is a drug.

18. The method of claim 17, wherein said drug is a cancer chemotherapeutic agent.

19. The method of claim 18, wherein said cancer chemotherapeutic agent is doxorubicin.

5 20. The method of claim 9, wherein said tumor homing molecule is an inhibitor of a CD13-like aminopeptidase.

21. The method of claim 20, wherein said inhibitor is selected from the group consisting of bestatin, actinonin and o-phenanthroline.

10 22. The method of claim 21, wherein said moiety is a cytotoxic agent.

23. The method of claim 21, wherein said moiety is a drug.

15 24. The method of claim 23, wherein said drug is a cancer chemotherapeutic agent.

25. The method of claim 24, wherein said cancer chemotherapeutic agent is doxorubicin.

20 26. A method of inhibiting angiogenesis in a tumor of a subject, comprising administering to the subject a conjugate comprising a moiety linked to a tumor homing molecule that exhibits specific binding to an NGR receptor, whereby the moiety is directed to angiogenic vasculature.

27. The method of claim 26, wherein said tumor homing molecule is a peptide containing the sequence NGR.

28. The method of claim 27, wherein said moiety is a cytotoxic agent.

5 29. The method of claim 27, wherein said moiety is a drug.

30. The method of claim 29, wherein said drug is a cancer chemotherapeutic agent.

10 31. The method of claim 30, wherein said cancer chemotherapeutic agent is doxorubicin.

32. The method of claim 27, wherein said tumor homing peptide comprises a sequence selected from the group consisting of CNGRCVSGCAGRC (SEQ ID NO:3), NGRAHA (SEQ ID NO:6), CVLNGRMEC (SEQ ID NO:7), and CNGRC (SEQ ID NO:8).

15 33. The method of claim 32, wherein said moiety is a cytotoxic agent.

34. The method of claim 32, wherein said moiety is a drug.

20 35. The method of claim 34, wherein said drug is a cancer chemotherapeutic agent.

36. The method of claim 35, wherein said cancer chemotherapeutic agent is doxorubicin.

37. The method of claim 26, wherein said tumor homing molecule is an inhibitor of a CD13-like aminopeptidase.

38. The method of claim 37, wherein said inhibitor is selected from the group consisting of bestatin, actinonin
5 and o-phenanthroline.

39. The method of claim 37, wherein said moiety is a cytotoxic agent.

40. The method of claim 37, wherein said moiety is a drug.

10 41. The method of claim 40, wherein said drug is a cancer chemotherapeutic agent.

42. The method of claim 41, wherein said cancer chemotherapeutic agent is doxorubicin.

43. A method of imaging the angiogenic vasculature of
15 a tumor in a subject, comprising:

(a) administering to the subject a conjugate comprising a detectable moiety linked to a tumor homing molecule that exhibits specific binding to an NGR receptor,
whereby said conjugate specifically binds said
20 angiogenic vasculature; and

(b) detecting said conjugate.

44. The method of claim 43, wherein said detectable moiety is a radionuclide.

45. The method of claim 43, wherein said tumor homing molecule is a peptide containing the sequence NGR.

46. The method of claim 45, wherein said tumor homing peptide comprises a sequence selected from the group
5 consisting of CNGRCVSGCAGRC (SEQ ID NO:3), NGRAHA (SEQ ID NO:6), CVLNGRMEC (SEQ ID NO:7), and CNGRC (SEQ ID NO:8).

47. The method of claim 46, wherein said detectable moiety is a radionuclide.

48. The method of claim 47, wherein said radionuclide
10 is selected from the group consisting of indium-111, technitium-99, carbon-11 and carbon-13.

49. The method of claim 43, wherein said tumor homing molecule is an inhibitor of a CD13-like aminopeptidase.

50. The method of claim 49, wherein said inhibitor is
15 selected from the group consisting of bestatin, actinonin and o-phenanthroline.

51. The method of claim 49, wherein said detectable moiety is a radionuclide.

52. The method of claim 51, wherein said radionuclide
20 is selected from the group consisting of indium-111, technitium-99, carbon-11 and carbon-13.

53. A substantially purified target molecule,
comprising an NGR receptor that binds a peptide comprising the sequence NGR, provided that said NGR receptor does not
25 have the amino acid sequence of CD13/aminopeptidase N (SEQ ID NO:201).

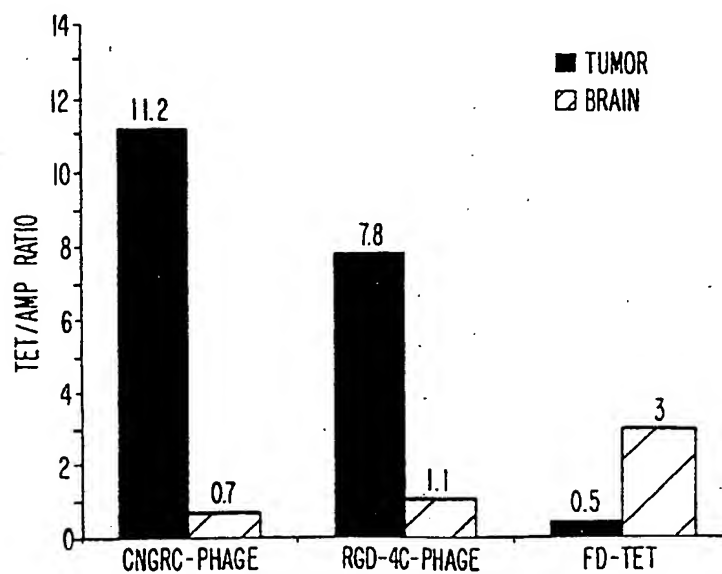
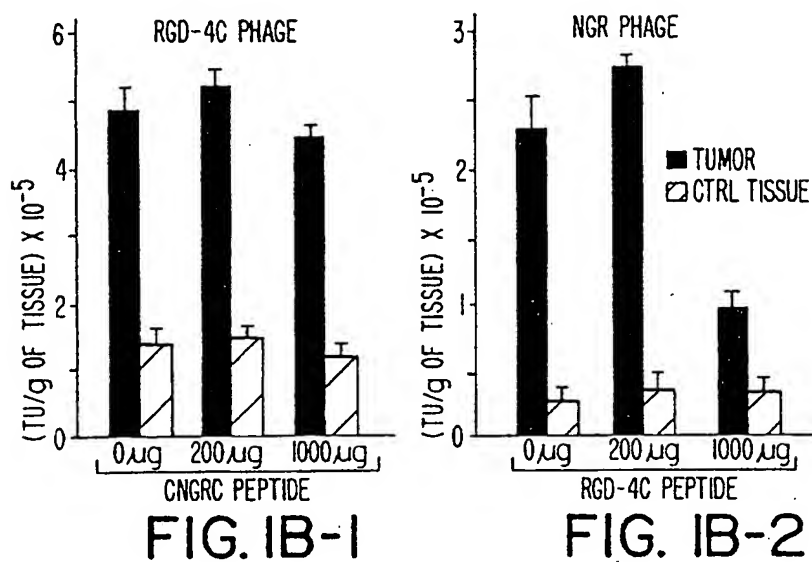
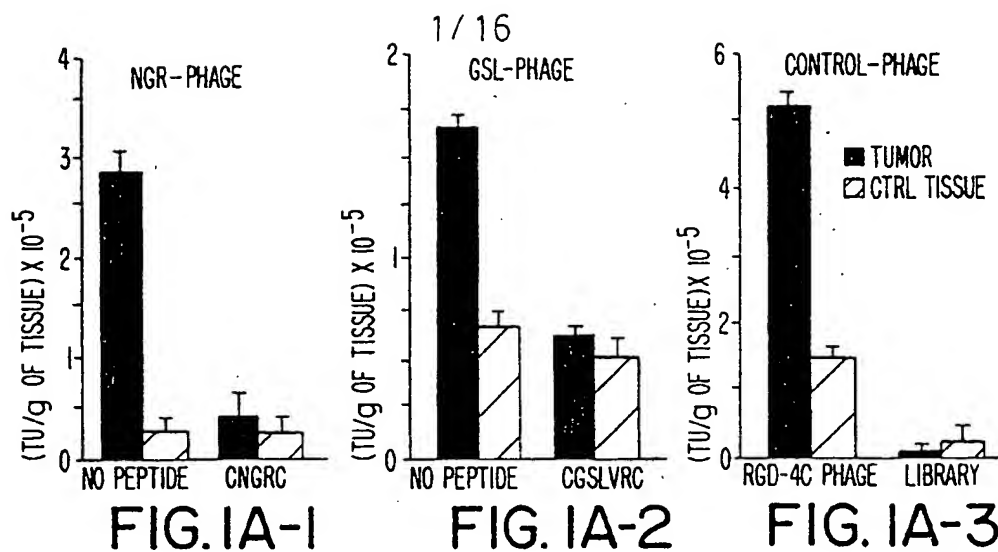


FIG. 2

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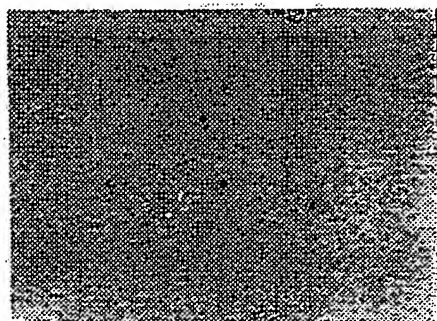


FIG. 3A

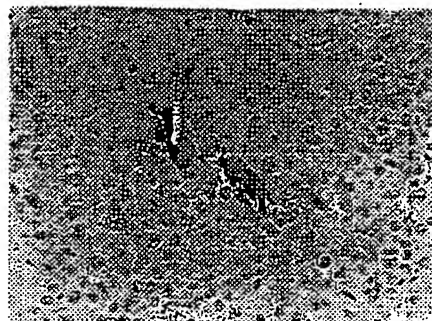


FIG. 3E

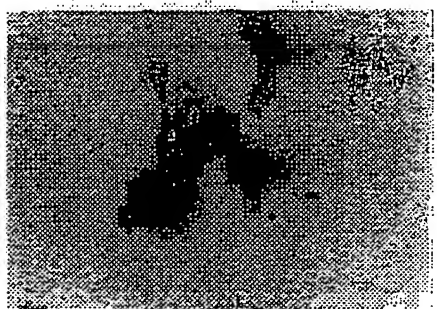


FIG. 3B



FIG. 3F

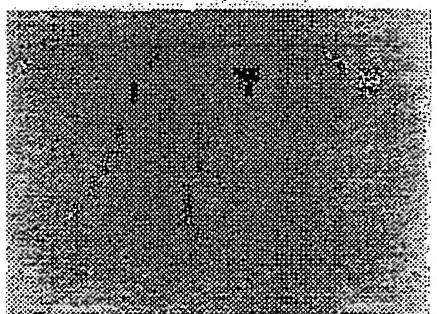


FIG. 3C

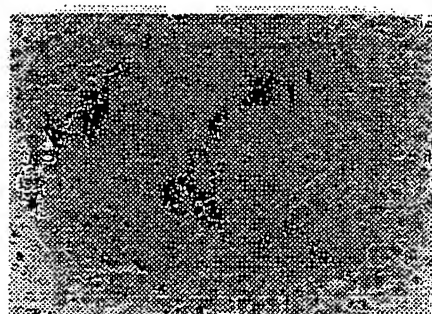


FIG. 3G

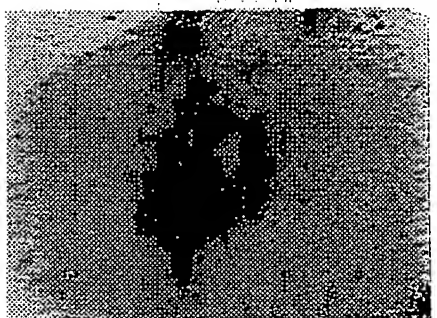


FIG. 3D

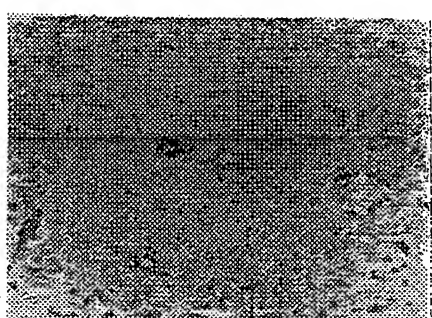


FIG. 3H

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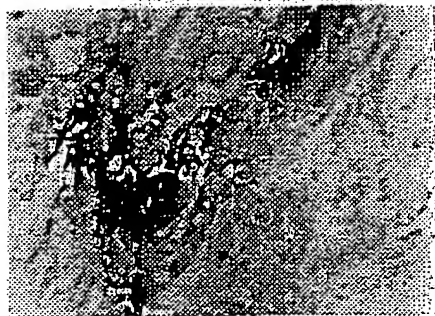


FIG. 3I

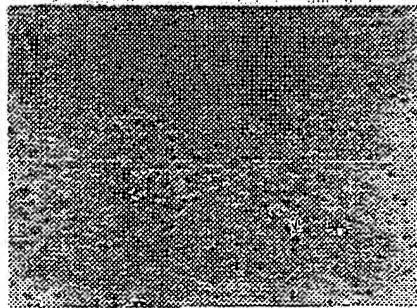


FIG. 3M

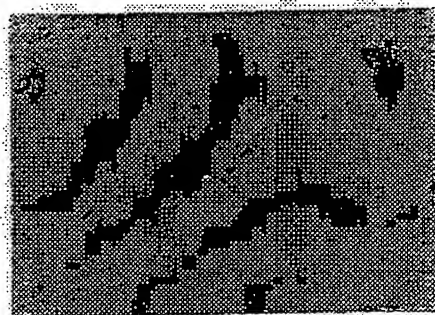


FIG. 3J

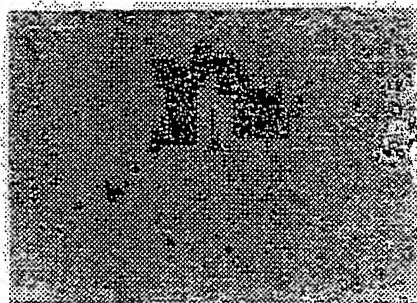


FIG. 3N

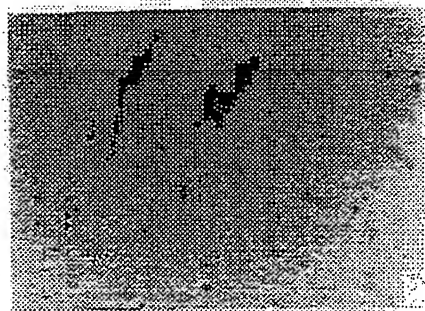


FIG. 3K

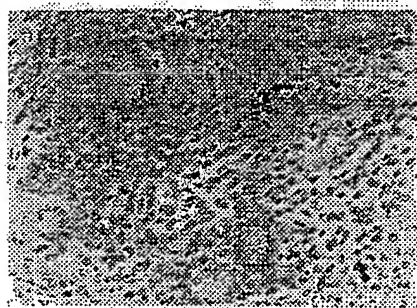


FIG. 3O

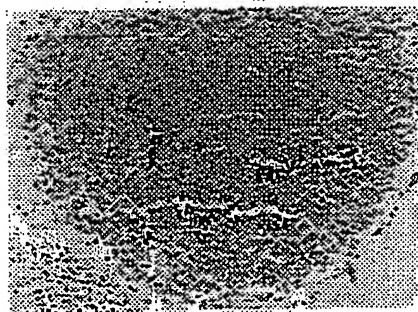


FIG. 3L

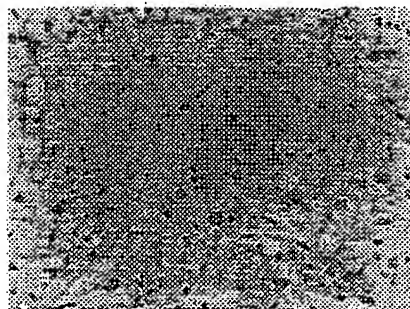


FIG. 3P

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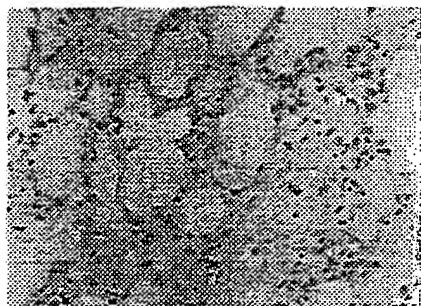


FIG. 3Q

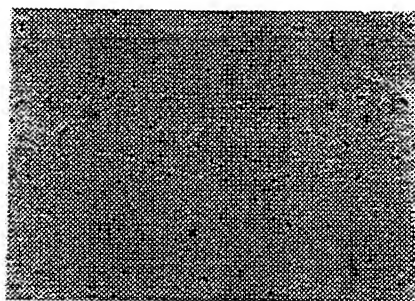


FIG. 3T

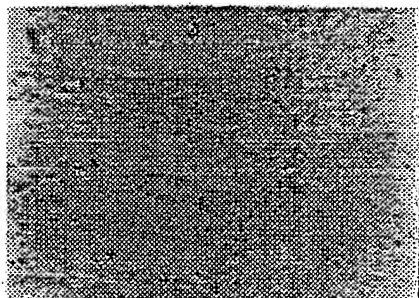


FIG. 3R

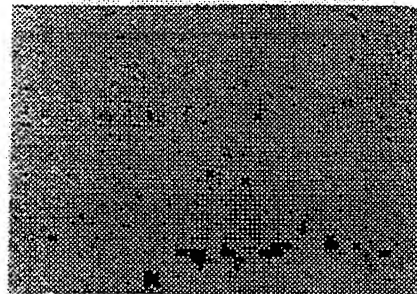


FIG. 3U

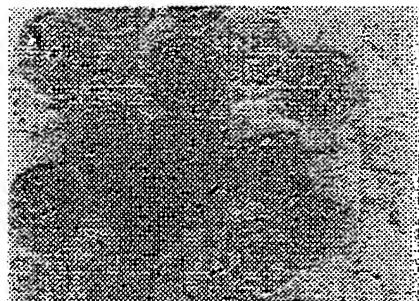


FIG. 3S

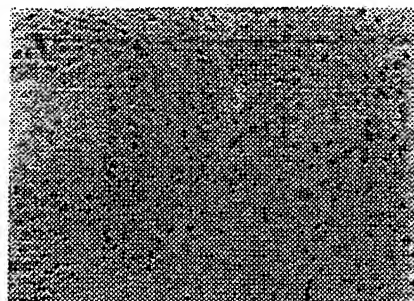


FIG. 3V

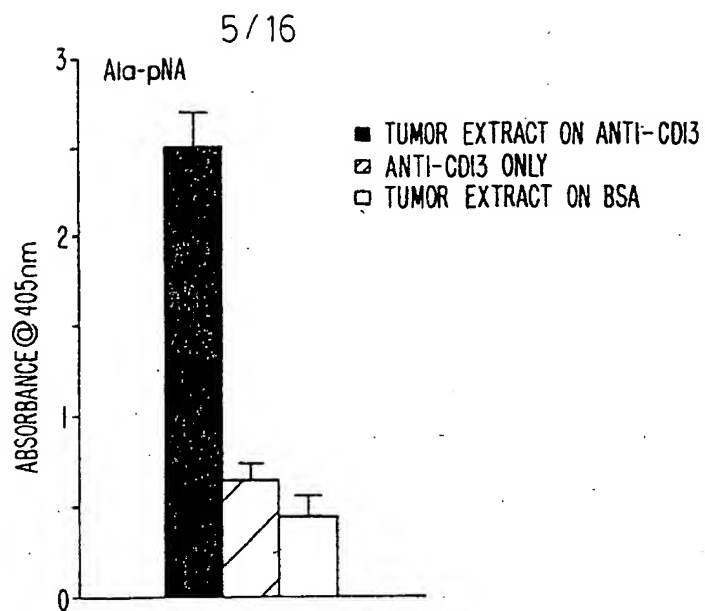


FIG. 4A

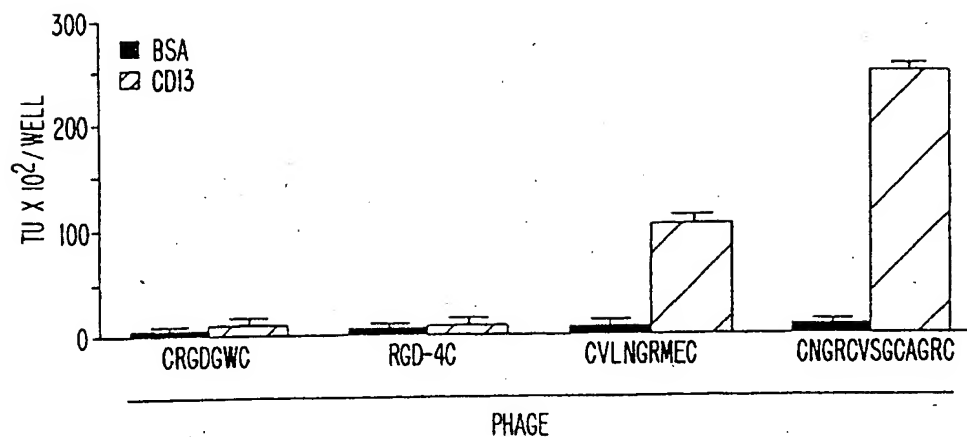


FIG. 4B

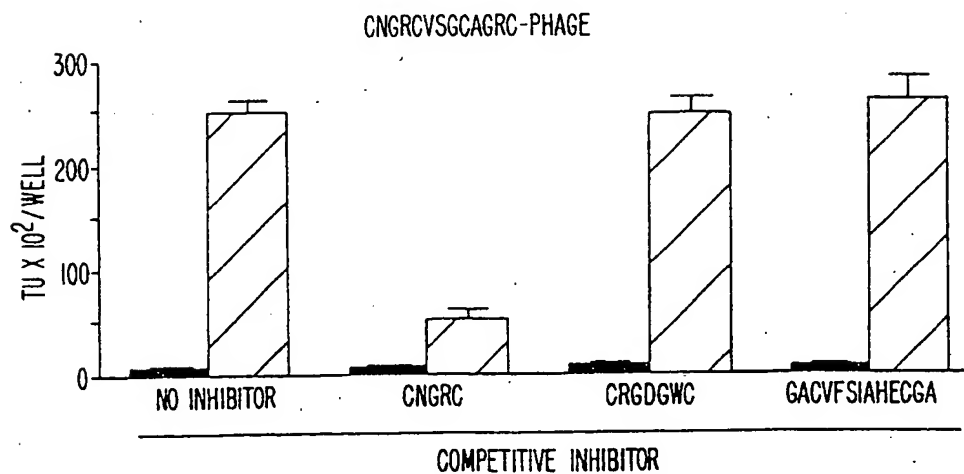


FIG. 4C

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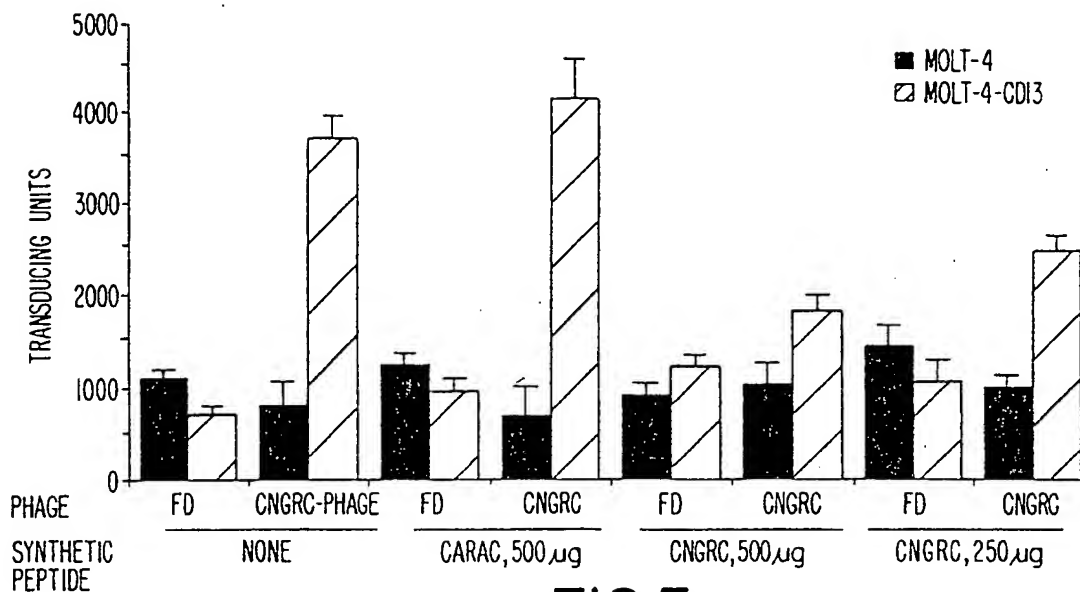


FIG.5

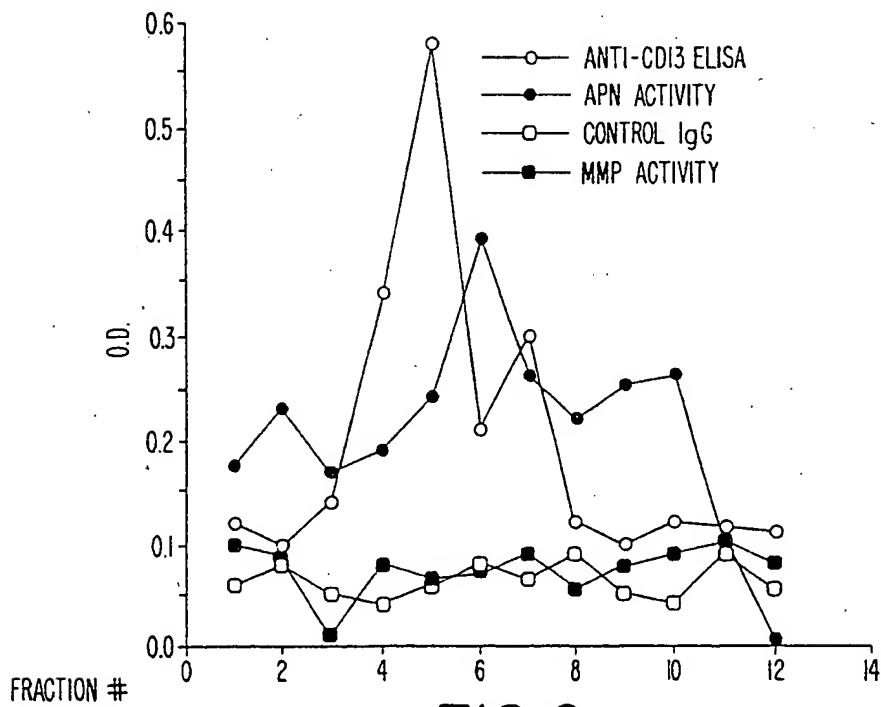


FIG.6

SUBSTITUTE SHEET (RULE 26)

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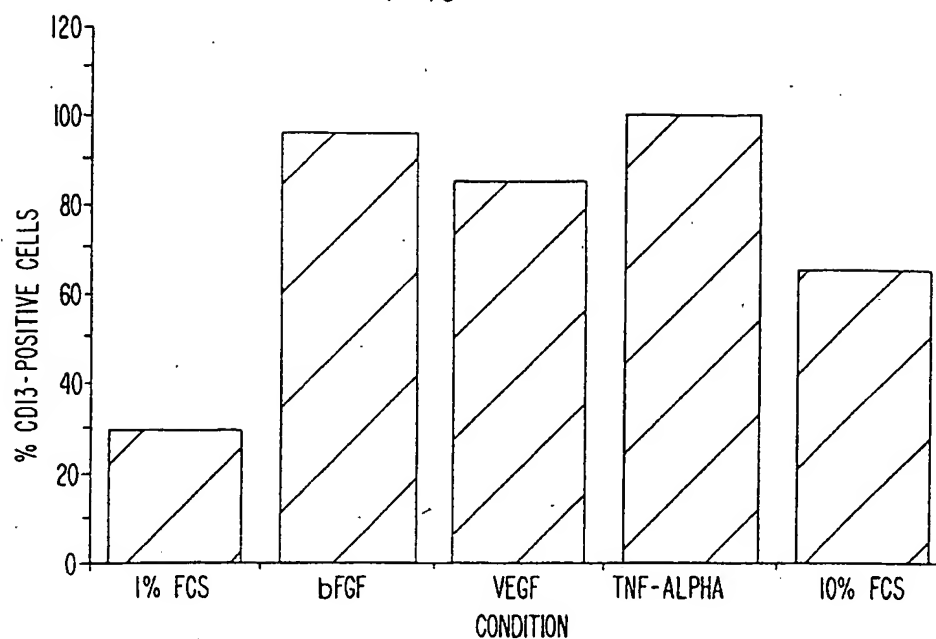


FIG. 7

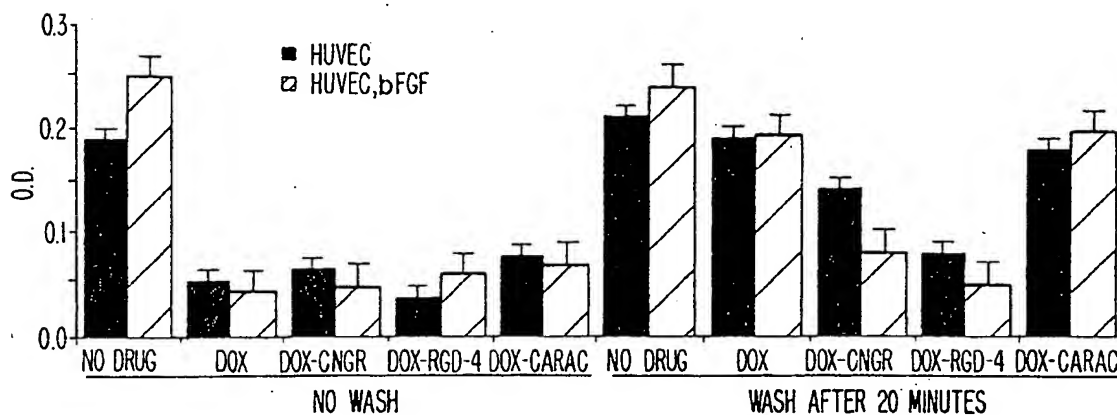


FIG. 8A

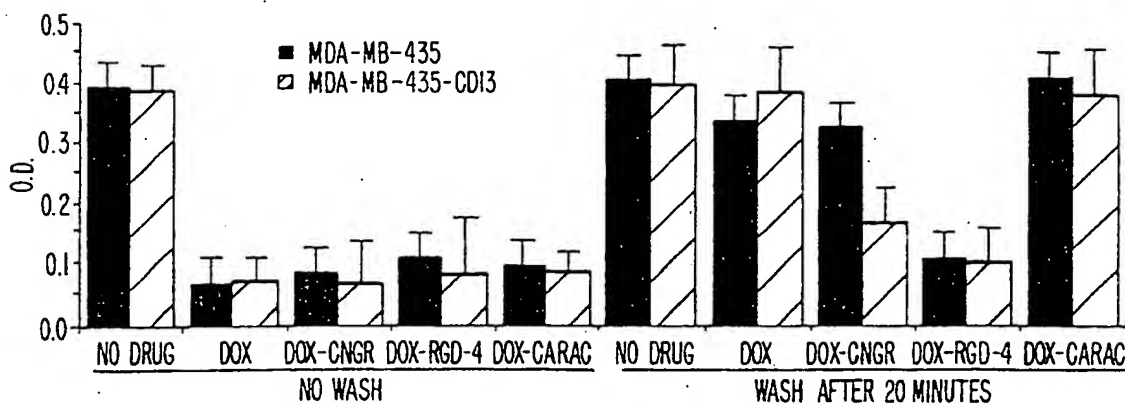


FIG. 8B

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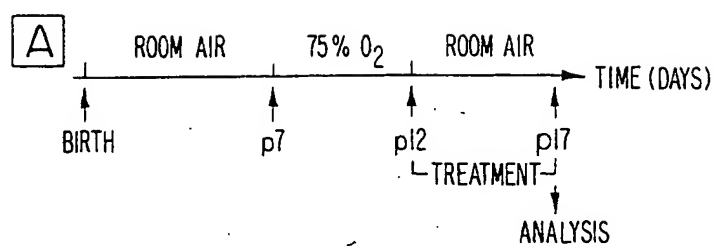


FIG. 9A

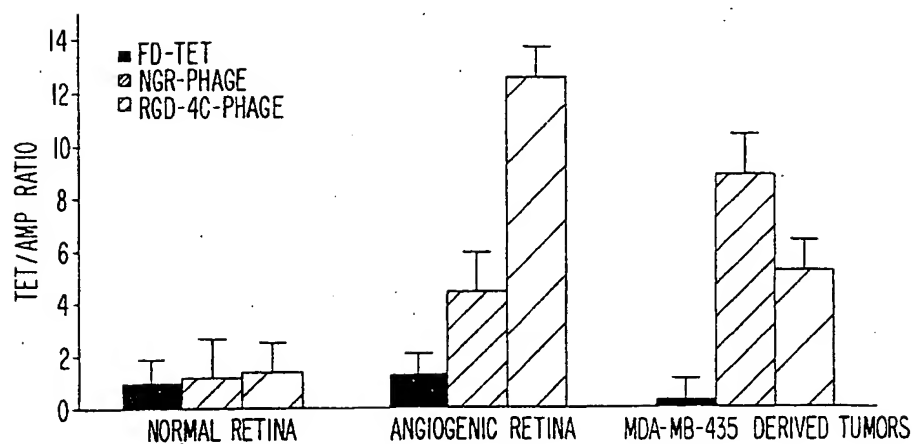


FIG. 9B

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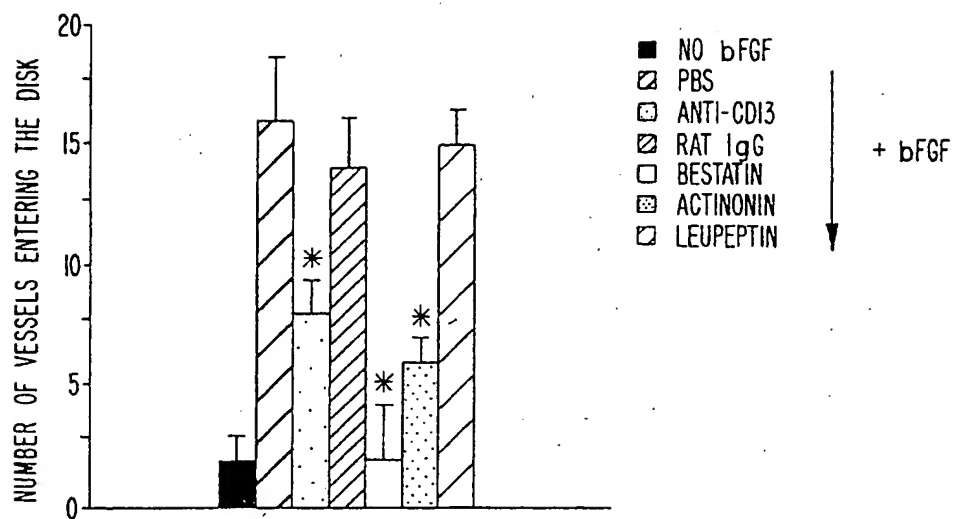


FIG. IOA

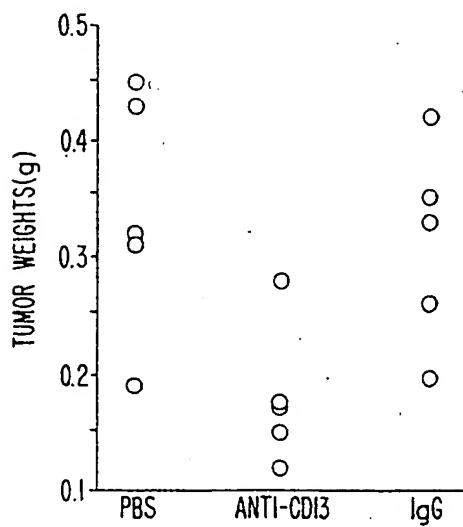


FIG. IOB

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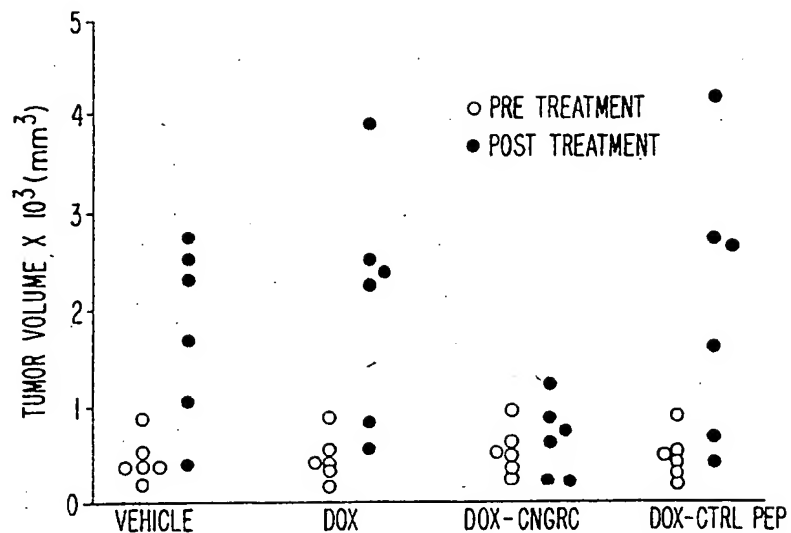


FIG. 1IA

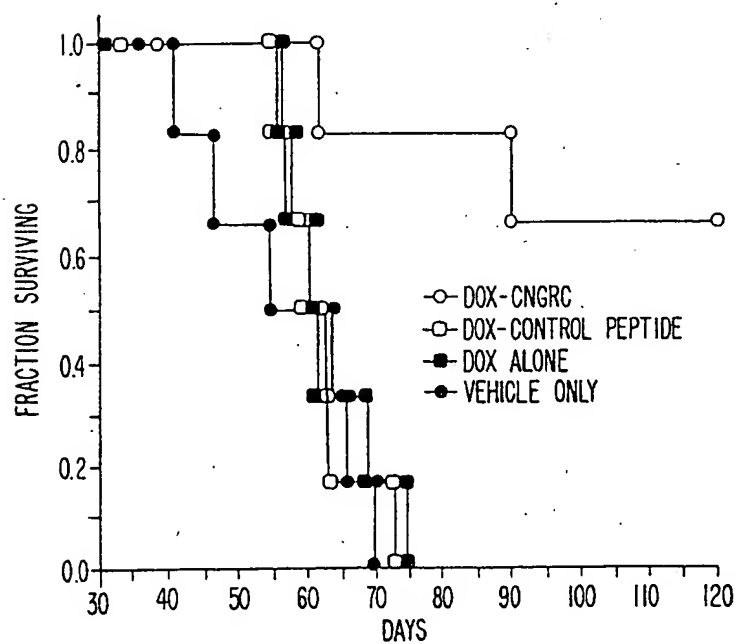


FIG. 1IB

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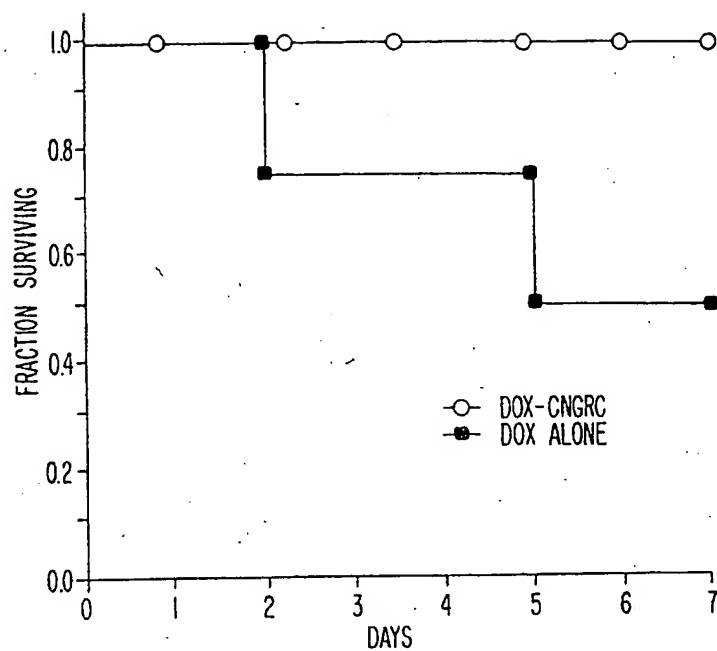


FIG. 1 IC

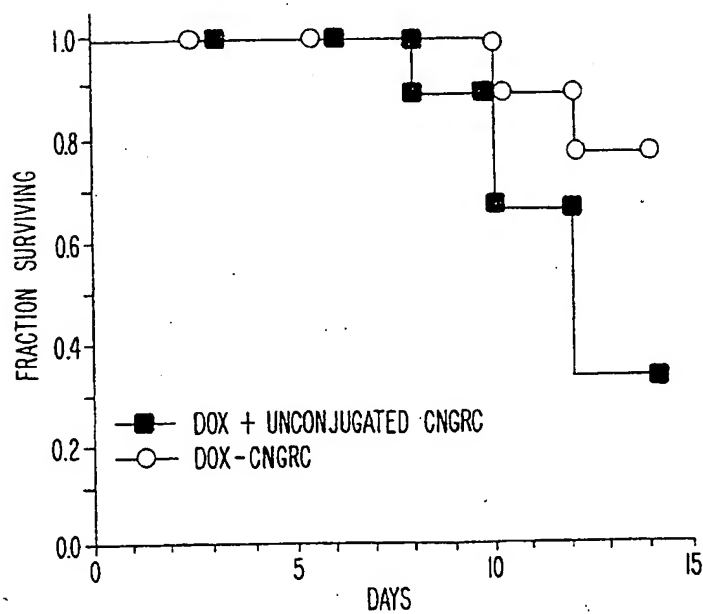


FIG. 1 ID

SUBSTITUTE SHEET (RULE 26)

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 1 MET Ala Lys Gly Phe Tyr Ile Ser Lys Ser Leu Gly Ile Leu Gly Ile Leu Leu Gly Val
 GCA GCC GTG TGC ACA ATC ATC GCA CTG TCA GTG GTG TAC TCC CAG GAG AAG AAC AAG AAC
 Ala Ala Val Cys Thr Ile Ile Ala Leu Ser Val Val Tyr Ser Gln Glu Lys Asn Lys Asn
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 41 Ala Asn Ser Ser Pro Val Ala Ser Thr Thr Pro Ser Ala Ser Ala Thr Thr Asn Pro Ala
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 Ser Ala Thr Thr Leu Asp Gln Ser Lys Ala Trp Asn Arg Tyr Arg Leu Pro Asn Thr Leu
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 61 AAA CCC GAT TCC TAC CAG GTG ACG CTG AGA CCG TAC CTC ACC CCC AAT GAC AGG GGC CTG
 81 Lys Pro Asp Ser Tyr Gln Val Thr Leu Arg Pro Tyr Tyr Leu Thr Pro Asn Asp Arg Gly Leu
 TAC GTT TTT AAG GGC TCC AGC ACC Thr Val Arg Phe Thr ACC TGC AAG GAG GCC ACT GAC GTC ATC
 Tyr Val Phe Lys Lys Gly Ser Ser Thr Val Arg Phe Thr [Cys] Lys Glu Ala Thr Asp Val Ile
 481 ATC ATC CAC AGC AAG AAG CTC AAC TAC ACC CTC AGC CAG GGG CAC AGG GTG GTC CTG CGT
 121 Ile Ile His Ser Lys Lys Leu Asn Tyr Thr Leu Ser Gln Gly His Arg Val Val Leu Arg
 GGT GTG GGA GGC TCC CAG CCC CCG GAC ATT GAC AAG ACT GAG CTG GTG GAG CCC ACC GAG
 Gly Val Gly Gly Ser Gln Pro Pro Asp Ile Asp Lys Thr Glu Leu Val Glu Pro Thr Glu
 601 TAC CTG GTG GTG CAC CTC AAG GGC TCC CTG GTG AAG GAC AGC CAG TAT GAG ATG GAC AGC
 161 Tyr Leu Val Val His Leu Lys Gly Ser Leu Val Lys Asp Ser Gln Tyr Tyr Glu Met Asp Ser
 GAG TTC GAG GGG GAG TTG GCA GAT GAC CTG GCG GGC TTC TAC CGC AGC GAG TAC ATG GAG
 Glu Phe Glu Gly Glu Leu Ala Asp Asp Leu Ala Gly Phe Tyr Arg Ser Glu Tyr Met Glu

FIG. 12A

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721 GGC AAT GTC AGA AAG GTG GTG GGC ACT ACA CAG ATG CAG GCT GCA GAT GCC CGG AAG TCC
 201 Gly Asn Val Arg Lys Val Val Ala Thr Thr Gln Met Gln Ala Ala Asp Ala Arg Lys Ser

 TTC CCA TGC Cys Phe Asp Glu GAG CCG GCC ATG AAG GCC GAG TTC AAC ATC ACG CTT ATC CAC CCC
 Phe Pro Phe Asp Glu Pro Ala Met Lys Ala Glu Phe Asn Ile Thr Leu Ile His Pro

 841 AAG GAC CTG ACA GCC CTG TCC AAC ATG CTT CCC AAA GGT CCC AGC ACC CCA CTT CCA GAA
 241 Lys Asp Leu Thr Ala Leu Ser Asn Met Leu Pro Lys Gly Pro Ser Thr Pro Leu Pro Glu

 GAC CCC AAC TGG AAT GTC ACT GAG TTC CAC ACC ACG CCC AAG ATG TCC ACG TAC TTG CTG
 Asp Pro Asn Trp Asn Val Thr Glu Phe His Thr Thr Pro Lys Met Ser Thr Tyr Leu Leu

 961 GCC TTC ATT GTC AGT AGT GAG TTC GAC TAC GTG GAG AAG CAG GCA TCC AAT GGT GTC TTG ATC
 281 Ala Phe Ile Val Ser Glu Phe Asp Tyr Val Val Glu Lys Gln Ala Ser Asn Gly Val Leu Ile

 CGG ATC TGG GCC CGG CCC AGT GCC ATT GCG GCG GGC CAC GGC GAT TAT GCC CTG AAC GTG
 Arg Ile Trp Ala Arg Pro Ser Ala Ile Ala Ala Gly His Gly Asp Tyr Ala Leu Asn Val

 1081 ACG GGC CCC ATC CTT AAC TTC TTT GCT GGT CAT TAT GAC ACA CCC TAC CCA CTC CCA AAA
 321 Thr Gly Pro Ile Leu Asn Phe Phe Ala Gly His Tyr Asp Thr Pro Tyr Pro Leu Pro Lys

 TCA GAC CAG ATT GGC CTG CCA GAC TTC AAC GCC GGC GCC ATG GAG AAC TGG GGA CTG GTG
 Ser Asp Gln Ile Gly Leu Pro Asp Phe Asn Ala Gly Ala Met Glu Asn Trp Gly Leu Val

 1201 ACC TAC CGG GAG AAC TCC CTG CTG TCC GAC CCC CTG TCC TCC AGC AGC AAC AAG GAG
 361 Thr Tyr Arg Glu Asn Ser Leu Leu Phe Asp Pro Leu Ser Ser Ser Ser Asn Lys Glu

 CGG GTG GTC ACT GTG ATT GCT CAT GAG CTG GGC CAC CAG TGG TTC GGG AAC CTG GTG ACC
 Arg Val Val Thr Val Ile Ala His Glu Leu Ala His Gln Trp Phe Gly Asn Leu Val Thr

 1321 ATA GAG TGG TGG AAT GAC CTG TGG TGG AAC GAG GGC TTC GCC TCC TAC GTG GAG TAC CTG
 401 Ile Glu Trp Trp Trp Asn Asp Leu Leu Trp Trp Phe Ala Ser Tyr Val Glu Tyr Leu

 GGT GCT GAC TAT GCG GAG CCC ACC TGG AAC TTG AAA GAC CTC ATG GTG CTG AAT GAT GTG
 Gly Ala Asp Tyr Ala Glu Pro Thr Trp Asn Leu Lys Asp Leu Met Val Leu Asn Asp Val

FIG. 12B

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1441 441	TAC CGC GTG ATG GCA GTG GAT GCA CTG GCC TCC TCC CAC CCG CTG TCC ACA CCC GCC TCG Tyr Arg Val Met Ala Val Asp Ala Leu Ala Ser Ser His Pro Leu Ser Thr Pro Ala Ser
1561 481	GAG ATC AAC ACG CCG GCC CAG ATC AGT GAG CTG TTT GAC GCC ATC TCC TAC AGC AAG GGC Glu Ile Asn Thr Pro Ala Gln Ile Ser Glu Leu Phe Asp Ala Ile Ser Tyr Ser Lys Gly
1561 481	GCC TCA GTC CTC AGG ATG CTC TCC AGC TTC CTG TCC GAG GAC GTA TTC AAG CAG GGC CTG Ala Ser Val Leu Arg Met Leu Ser Ser Phe Leu Ser Ser Glu Asp Val Phe Lys Gln Gly Leu
1681 521	GCG TCC TAC CTC CAC ACC TTT GCC TAC CAG AAC ACC ATC TAC CTG AAC CTG TGG GAC CAC Ala Ser Tyr Leu Leu His Thr Phe Ala Tyr Gln Asn Thr Ile Tyr Leu Asn Leu Trp Asp His
1681 521	CTG CAG GAG GCT GTG AAC AAG CGG TCC ATC CAA CTC CCC ACC ACC GTG CGG GAC ATC ATG Leu Gln Glu Ala Val Asn Asp Arg Ser Ile Gln Leu Pro Thr Thr Val Arg Asp Ile Met
1801 561	AAC CGC TGG ACC CTG CAG ATG GGC TTC CCG GTC ATC ACG GTG GAT ACC ACC GTG GAT ACC ACC Asn Arg Trp Thr Thr Leu Gln Met Gly Phe Pro Val Ile Thr Val Val Asp Thr Ser Thr Gly Thr
1801 561	CTT TCC CAG GAG CAC TTC CTC CTT GAC CCC GAT TCC AAT GTT ACC ACC CGC CCC TCA GAA TTC Leu Ser Gln Glu His Phe Leu Leu Asp Pro Asp Ser Asn Val Thr Arg Pro Ser Glu Phe
1921 601	AAC TAC GTG TGG ATT GTG CCC ATC ACA TCC ATC AGA GAT GGC AGA CAG CAG GAC TAC Asn Tyr Val Trp Ile Val Pro Ile Thr Ser Ile Arg Asp Gly Arg Gln Gln Asp Tyr
1921 601	TGG CTG ATA GAT GTA AGA GCC CAG AAC GAT CTC TTC AGC ACA TCA GGC AAT GAG TGG GTC Trp Leu Ile Asp Val Arg Ala Gln Asn Asp Leu Phe Ser Thr Ser Gly Asn Glu Trp Val
2041 641	CTG CTG AAC CTC AAT GTG ACG GGC TAT TAC CGG GTG AAC TAC GAC GAA GAG AAC TGG AGG Leu Leu Asn Leu Asn Val Thr Gly Tyr Tyr Arg Val Asn Tyr Asp Glu Glu Asn Trp Arg
2041 641	AAG ATT CAG ACT CAG CTG CAG AGA GAC CAC TCG GCC ATC CCT GTC ATC AAT CGG GCA CAG Lys Ile Gln Thr Gln Leu Gln Arg Asp His Ser Ala Ile Pro Val Ile Asn Arg Ala Gln
2041 641	ATC ATT AAT GAC GCC TTC AAC CTG GCC AGT GCC CAT AAG GTC CCT GTC ACT CTG GCG CTG Ile Ile Asn Asp Ala Phe Asn Leu Ala Ser Ala His Lys Val Pro Val Thr Leu Ala Leu

FIG. 12C

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2161 681	AAC AAC ACC CTC TTC CTG ATT GAA GAG AGA CAG TAC ATG CCC TGG GAG GCC GCC CTG AGC Asn Asn Thr Leu Phe Leu Ile Glu Glu Arg Gln Tyr Met Pro Trp Glu Ala Ala Leu Ser
	AGC CTG AGC TAC TTC AAG CTC ATG TTT GAC CGC TCC GAG GTC TAT GGC CCC ATG AAG AAC Ser Leu Ser Tyr Phe Phe Met Phe Asp Arg Ser Ser Glu Val Tyr Gly Pro Met Lys Asn
2281 721	TAC CTG AAG AAG CAG GTC ACA CCC CTC TTC ATT CAC TTC AGA AAT ACC AAC AAC TGG Tyr Leu Lys Lys Gln Val Thr Pro Leu Phe Ile His Phe Arg Asn Thr Asn Asn Trp
	AGG GAG ATC CCA GAA AAC CTG ATG GAC CAG TAC AGC GAG GTT AAT GCC ATC AGC ACC GCC Arg Glu Ile Pro Glu Asn Leu Met Asp Gln Tyr Ser Ser Glu Val Asn Ala Ile Ser Thr Ala
2401 761	TGC TCC AAC GGA GTT CCA GAG TGT GAG GAG ATG GTC TCT GGC CTT TTC AAG CAG TGG ATG Cys Ser Asn Gly Val Pro Glu Cys Glu Glu Met Val Ser Gly Leu Phe Lys Gln Trp Met
	GAG AAC CCC AAT AAT AAC CCG ATC CAC CCC AAC CTG CGG TCC ACC GTC TAC TGC AAC GCT Glu Asn Pro Asn Asn Asn Pro Ile His Pro Asn Leu Arg Ser Thr Val Cys Asn Ala
2521 801	ATC GCC CAG GGC GGG GAG GAG GAG TGG GAC TTC GCC TGG GAG CAG TTC CGA AAT GCC ACA Ile Ala Gln Gly Gly Glu Glu Glu Trp Asp Phe Ala Trp Glu Gln Phe Arg Asn Ala Thr
	CTG GTC AAT GAG GCT GAC AAG CTC CGG GCA GCC CTG GCC TGC AGC AAA GAG TTG TGG ATC Leu Val Asn Asn Glu Ala Asp Lys Leu Arg Ala Ala Leu Ala Cys Ser Lys Glu Leu Trp Ile
2641 841	CTG AAC AGG TAC CTG AGC TAC ACC CTG AAC CCG GAC TTA ATC CGG AAG CAG GAC GCC ACC Leu Asn Arg Tyr Leu Ser Tyr Thr Leu Asn Pro Asp Leu Ile Arg Lys Gln Asp Ala Thr
	TCT ACC ATC ATC AGC ATT ACC AAC AAG GTC ATT GGG CAA GGT CTG GTC TGG GAC TTT GTC Ser Thr Ile Ile Ser Ile Thr Asn Asn Val Ile Gly Gln Gly Leu Val Trp Asp Phe Val
2761 881	CAG AGC AAC TGG AAG AAG CTT TTT AAC GAT TAT GGT GGT GGC TCG TTC TCC TCC AAC Gln Ser Asn Trp Lys Lys Leu Phe Asn Asp Tyr Gly Gly Ser Phe Ser Phe Ser Asn
	CTC ATC CAG GCA GTG ACA CGA TCC ACC GAG TAT GAG CTG CAG CTG GAG CAG Leu Ile Gln Ala Val Thr Arg Arg Phe Ser Thr Glu Tyr Glu Leu Gln Gln Leu Glu Gln

FIG. 12D

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2881 TTC AAG AAG GAC AAC GAG GAA ACA GGC TTC GGC TCA GGC ACC CGG GCC CTG GAG CAA GCC
 921 Phe Lys Lys Asp Asn Glu Glu Thr Gly Phe Gly Ser Gly Thr Arg Ala Leu Glu Gln Ala

 CTG GAG AAG ACG AAA GCC AAC ATC AAG TGG GTG AAG GAG AAC AAG GAG GTG GTG CTC CAG
 Leu Glu Lys Thr Lys Ala Asn Ile Lys Trp Val Lys Glu Asn Lys Glu Val Val Leu Gln

 3001 TGG TTC ACA GAA AAC AGC AAA TAGTCCCCA GCCCTTGAAG TCACCCCGGCC CCGATGCAAG GTGCCCCAC
 961 Trp Phe Thr Glu Asn Ser Lys

 AT GTGTCCATCC CAGCGGCTGG TGCAGGGCCT CCATTCTCTGG AGCCCGAGGC

 3121 ACCAGTGTCC TCCCCTCAAG GACAAAGTCT CCAGCCCCAGG TTCCTCTGCTGCTGAGGCCA GTCTAGTTCC TG

 ATGACCCA GCGTGCCTGA GCACCTCCCCA GCCCCTGCCC CTCATGCCAA

 3241 CCCC GCCCTA GGCCTGGCAT GGCACCTGTC GCCCAGTGCC CTGGGGCTGA TCTCAGGGAA GCCCAGCTCC AG

 GGCCAGAT GAGCAGAAGC TCTCGATGGA CAATGAACGG CCTTGCTGGG

 3361 GGCCGCCCTG TACCCTCTTT CACCTTTCCC TAAAGACCCT AAATCTGAGG AATCAACAGG GCAGCAGATC TG

 TATATTTT TTCTAAGAG AAAATGTAAA TAAAGGATTT CTAGATGAAA

 3481 AAAAAAAAAA AAAA

FIG. 12E

INTERNATIONAL SEARCH REPORT

Int. l. Application No

PCT/US 98/18895

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/50 G01N33/574

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P, L	WO 98 10795 A (BURNHAM INST) 19 March 1998	9-19
X	see claims 55-66 see page 9, line 15 - line 18 see page 35, line 35 - page 36, line 12 see page 37, line 5 - page 39, line 12 see page 66, line 24 - page 67, line 16 L, Priority: first filing of invention. (Paris Convention, Art. 4C(4)). --- -/--	1-9, 20-53

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

*** Special categories of cited documents :**

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

2 February 1999

Date of mailing of the international search report

16/02/1999

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Hoekstra, S

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/18895

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>KOIVUNEN E ET AL: "PHAGE LIBRARIES DISPLAYING CYCLIC PEPTIDES WITH DIFFERENT RING SIZES: LIGAND SPECIFICITIES OF THE RGD-DIRECTED INTEGRINS" BIO/TECHNOLOGY, vol. 13, no. 3, March 1995, pages 265-270, XP002055920 see table 1</p> <p style="text-align: center;">----</p>	1,2,4-6, 8-53
X	<p>ERKKI KOIVUNEN ET AL: "SELECTION OF PEPTIDES BINDING TO THE X5B1 INTEGRIN FROM PHAGE DISPLAY LIBRARY*" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 27, 25 September 1993, pages 20205-20210, XP000396659 see page 20207, left-hand column - right-hand column</p> <p style="text-align: center;">----</p>	1,2,4-6, 8-53
X	<p>US 5 536 814 A (RUOSLAHTI ERKKI ET AL) 16 July 1996 cited in the application see column 6, line 64 - line 67</p> <p style="text-align: center;">----</p>	53
A	<p>WO 97 10507 A (JOLLA CANCER RES FOUND) 20 March 1997 see claims</p> <p style="text-align: center;">----</p>	1-53
A	<p>WO 95 14714 A (JOLLA CANCER RES FOUND) 1 June 1995</p> <p style="text-align: center;">-----</p>	1-53

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/18895

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210
2. ☒ Claims Nos.: 53 PARTIALLY
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees:

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Although claims 26-52 are directed to a method of treatment of the human/animal body, or diagnosis practised on the human/animal body the search has been carried out and based on the alleged effects of the conjugates.

Rule 39.1(iv) PCT - Diagnostic method practised on the human or animal body

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

CLAIMS Nos: 53 Partially

The protection to be conferred to the subject-matter of claim 53 extends to any molecule that binds molecules having the NGR motif. No technical features of the claimed matter is provided, other than a disclaimer, which describe the claimed molecule itself and which could form the basis for a meaningful search. Moreover, documents identified can not be tested for falling under the protection of present claim 53. Thus, due to non-compliance with the prescribed requirements (Article 6, Rule 6.3(a) PCT), a meaningful search for the subject-matter of claim 53 is not possible.

Documents which provided information of molecules as to their binding ability towards the NGR motif have been cited.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Although claims 26-52 are directed to a method of treatment of the human/animal body, or diagnosis practised on the human/animal body the search has been carried out and based on the alleged effects of the conjugates.

Rule 39.1(iv) PCT - Diagnostic method practised on the human or animal body

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

Claims Nos.: 53 PARTIALLY

The protection to be conferred to the subject-matter of claim 53 extends to any molecule that binds molecules having the NGR motif. No technical features of the claimed matter is provided, other than a disclaimer, which describe the claimed molecule itself and which could form the basis for a meaningful search. Moreover, documents identified can not be tested for falling under the protection of present claim 53. Thus, due to non-compliance with the prescribed requirements (Article 6, Rule 6.3(a) PCT), a meaningful search for the subject-matter of claim 53 is not possible.

Documents which provided information of molecules as to their binding ability towards the NGR motif have been cited.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/18895

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9810795 A	19-03-1998	AU 4412297 A	02-04-1998
US 5536814 A	16-07-1996	US 5397362 A	14-03-1995
WO 9710507 A	20-03-1997	US 5622699 A	22-04-1997
		AU 6374098 A	18-06-1998
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		AU 1259695 A	13-06-1995
		EP 0730607 A	11-09-1996
		JP 9509142 T	16-09-1997
		US 5627263 A	06-05-1997